Association Study of Human Papillomavirus Infection in HIV Patients of Selected Pakistani Population

By
Areej Abdul Sattar
141-FBAS/MSBT/F14

Department of Bioinformatics and Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University Islamabad
2016

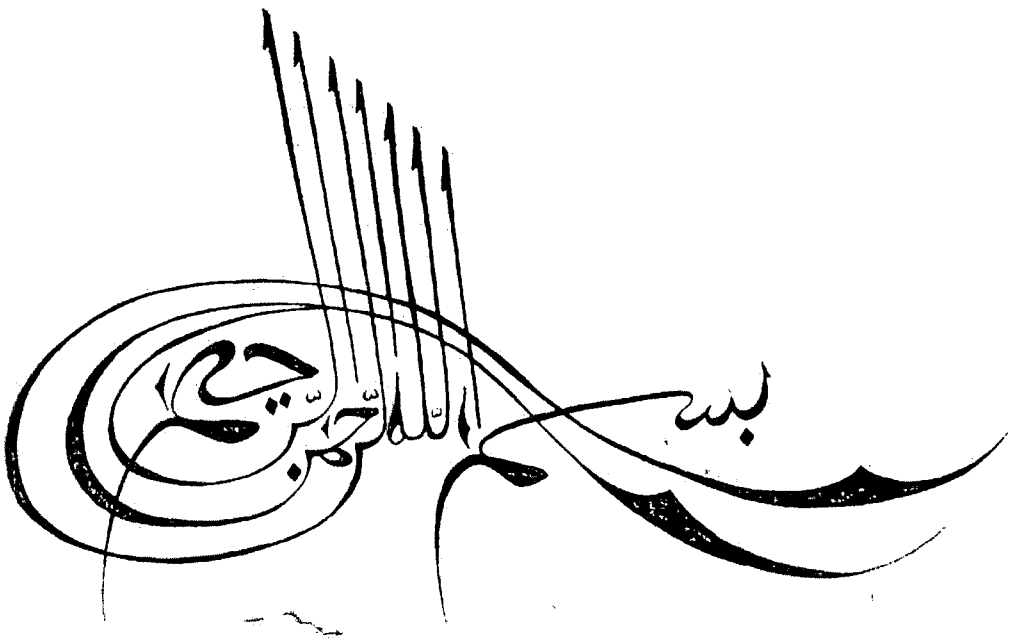
Association Study of Human Papillomavirus Infection in HIV Patients of Selected Pakistani Population

By
Areej Abdul Sattar
141-FBAS/MSBT/F14

Supervisor
Dr. Sobia Tabassum
IIUISenior scientist

Co-Supervisor
Dr. Hafsa AzizBI & BT Dept.
NORI Islamabad

Department of Bioinformatics and Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University Islamabad
2016


بسم الله الرحمن الرحيم

Dated: 10th Nov '16

FINAL APPROVAL

It is certified that we have read the thesis submitted by Areej Abdul Sattar 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.

COMMITTEE

External Examiner

Internal Examiner

Supervisor

Dr. Sobia Tabassum
Assistant Professor
Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Co-supervisor

Dr. Hafsa Aziz
Senior Scientist
Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad

Chairperson

Dean, FBAS

A thesis submitted to Department of Biotechnology & Bioinformatics,
FBAS,
International Islamic University, Islamabad
as a partial fulfilment of requirement for the award of the degree of MS
Biotechnology

Dedicated to

My Parents

Whose Love and Prayers accompanied me forever

And

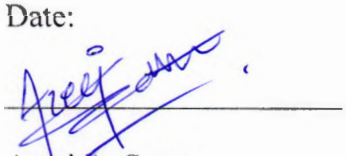
My Brothers and Sister

For their unwavering support

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: 29-Nov '16



Areej A. Sattar

TABLE OF CONTENTS

ACKNOWLEDGEMENT

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.

I am thankful to my family, my parents for their prayers and blessings and unconditional love and my siblings for their unwavering support throughout my project work.

I would like to thank my supervisor, Dr. Sobia Tabassum and co-supervisor Dr. Hafsa Aziz for their untoward support, keen interest and guidance throughout my research work.

I would like to thank Dr. Javaid Irfan (ex-Director NORI) for granting me a permission of working at Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad. I would also take an opportunity to thank Dr. Mohammad Faheem, (Director NORI) to allow me continue my research in this institute. Owing to this milieu, eventually I was able to accomplish my MS degree.

I express a deep feeling of respect for employees at NORI, Nargis Khatoon, and Noreen Kishwar for their valuable assistance at different occasions during

my entire research work. A special thanks to Dr. Sanam and Sister Rukhsana at PIMS for arranging and providing us with required samples for the study.

I would also like to thank my friends who cherish sincere and honest wishes for me in their hearts. I specially want to thank Huma Iqbal for being a great company, a friend and a sister and for her assistance and help throughout my project.

Areej Abdul Sattar

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
BSA	Bovine serum albumin
CIN I	Cervical intraepithelial neoplasia
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
E	Early region
FDA	Food and Drug Administration
FSW	Female sex workers
HGSIL	High-grade squamous intraepithelial lesions
HIV	Human Immunodeficiency virus
HPV	Human Papillomavirus
HR	High Risk
IARC	International association for research on cancer
IUD	Intra-uterine devices
L1	Late region 1
LBC	Liquid based cytology
LGSIL	Low-grade squamous intraepithelial lesions
LR	Low Risk
NORI	Nuclear oncology and radiotherapy institute
OC	Oral contraceptives
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PIMS	Pakistan Institute of Medical Sciences
RBC	Red blood cell
RNA	Ribonucleic acid
STD	Sexually transmitted disease
STI	Sexually transmitted infection
TBE	Tris/Borate/EDTA
UNAIDS	Joint United Nations Programme on HIV/AIDS
URR	Upstream regulatory region
WHO	World Health Organization

List of Figures

List of Tables

ABSTRACT

Human papillomavirus (HPV) infections play an important role in the pathogenesis of anogenital cancer and its precursors. HPV are the most prevalent sexually transmitted agents worldwide. Rates of cervical human papillomavirus (HPV) infection and abnormal cytology are significantly high in HIV infected women. Human immunodeficiency virus who are HPV infected are significantly more likely to have cervical cancer than the HPV infected general population of women. HIV and HPV continue to be epidemic of startling proportions in all around the world and in Pakistan. More than hundred subtypes of HPV are known till date. Among them HPV Type 16 and Type 18 are considered to be High risk types along with Type 33, Type 45 and Type 31. Thus, baseline information on human papillomavirus (HPV) prevalence and type distribution in HIV positive females is highly desirable to evaluate the association of HPV and HIV infections.

In the current study, general consensus primers were used to screen 50 samples (*n=50*) obtained from patients attending the special clinic for HIV patients in PIMS were screened for the presence of Human Papillomavirus and its subtypes. All the patients enrolled were HIV positive 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 50 patients (n=50), 30 (n=30) were found to be positive for Human Papillomavirus, indicating a prevalence of 60% in the population under study. Out of these 30 HPV positive patients, 9 (18%) were found to be high risk HPV 16, 5 (10%) were found to be high risk HPV 18, 4 (8%) were HPV type 33 and 2 (4%) were HPV type 45. The remaining 11 (22%) positive samples were low risk HPV subtypes. The obtained results were evaluated on the basis of different risk factors associated. These results indicate that Human Papillomavirus is highly prevalent in the HIV positive patients in Pakistani population, thus proper methods should be devised for the prevention and diagnosis of infection and its treatment.

Chapter

1. Introduction

Cervical cancer is the cancer rising from the abnormal growth of cervix's cells. The cervix is secured with a layer of skin like cells on its external surface, called the ectocervix. There are glandular cells covering within the cervix (the endocervix). Mucus is produced by these cells. The skin like cells of the ectocervix can get to be malignant, prompting a squamous cell cervical disease. Alternately the glandular cells of the endocervix can get to be destructive, prompting an adenocarcinoma of the cervix. Cervical cancer is considered the fourth most common type of cancer that is cause of mortality in females, worldwide. 528,000 new cases and 266,000 deaths were estimated in 2012 (Ferlay et al. 2015). Human Papillomavirus is the causative agent of cervical malignancy which can be easily sexually transmitted from person to person. 150 related viruses are grouped into HPV, out of them 40 of these types can be transmitted by the direct skin to skin contact, causing genital warts or invasive cancers. High-risk HPV types such as HPV16, 18, 31, 33 or 45 infection is involved in the development of cervical cancer (Woodman et al. 2007). Cervical biopsies showed the presence of HPV DNA in which HPV 16 and HPV 18 were more prevalent (Munoz et al. 2006). Whereas low risk types such as HPV 6 and 11 are associated with the genital condyloma or genital warts (D'Abramo et al. 2011).

There are certain risk factors that are key elements for the development of cancer in the HPV presence. These include polygamy, early age sexual activity, sexually transmitted diseases (STD), family history with cervical cancer, unusual Pap smear, smoking, immunosuppression due to HIV/AIDS and prolong corticosteroid use.

Human papillomavirus incorporate 40 firmly relevant however hereditarily particularly sexually transmitted infections which are normally classified as high risk or low risk as indicated by their oncogenic potential (de Villiers et al. 2004; Munoz et al. 2003; Munoz et al. 2006). Widely held HPV contaminations do not result in cancer. But still there are certain HPV types that have oncogenic potential to cause cervical cancer. Many epidemiological studies have shown that HR types of human papillomavirus are the causal agent of cervical tumor development, anogenital cancer and oral cavity and

oropharyngeal cancers (Parkin et al. 2006; Koutsky et al. 1997; Kreimer et al. 2005). 70% of cervical malignancies indicated the presence of HPV 16 and HPV 18 alone (Parkin et al. 2006). Whereas 90% of anogenital warts indicated the low risk types, including HPV 6 and HPV 11 (Lacey et al. 2006; D'Abramo et al. 2011). Since 2006, a quadrivalent vaccine against HPV types 6, 11, 16, and 18 has been recommended in the United States for routine use in 11 or 12 year old females with catch-up through 26 years of age for the prevention of cervical cancer and anogenital warts (Markowitz et al. 2007). In October 2009, a bivalent vaccine against HPV types 16 and 18 was licensed for routine use in females 9–26 years of age. Either vaccine is now recommended for use in females. Also in October 2009, the quadrivalent vaccine received US Food and Drug Administration approval for prevention of genital warts in males aged 9–26 years (Hariri et al. 2011).

1.1 Worldwide Human Papillomavirus Prevalence

Cervical growth is the fourth most normal tumor in ladies, and the seventh general, with an expected 528,000 new cases in 2012. Just like liver cancer, the global burden (around 85%) occurs in underdeveloped countries. According to the estimated data, prevalence of cervical malignancy is lowest in Australia/New Zealand (5.5%) and Western Asia (4.4%). High prevalent regions include Eastern Africa (42.7%), Melanesia (33.3%), Southern Africa (31.5%) and Middle Africa (30.6%). Cervical cancer is most commonly found in Eastern and Middle African women. In 2012, 266,000 mortalities were estimated which represented 7.5% of all the deaths caused by cancer in females. In underdeveloped countries, 9 out of 10 (87%) females dies of cervical cancer. Mortality fluctuates 18 fold within the diverse districts of the world, with rates running from under 2 for every 100,000 in Western Europe, Western Asia and Australia/New Zealand whereas over 20 for each 100,000 in Melanesia (20.6%), Middle Africa (22.2%) and Eastern Africa (27.6%) (Figure 1.1) (GLOBOCAN 2012 (IARC), Section of Cancer Surveillance (23/4/2016) WHO, 2015).

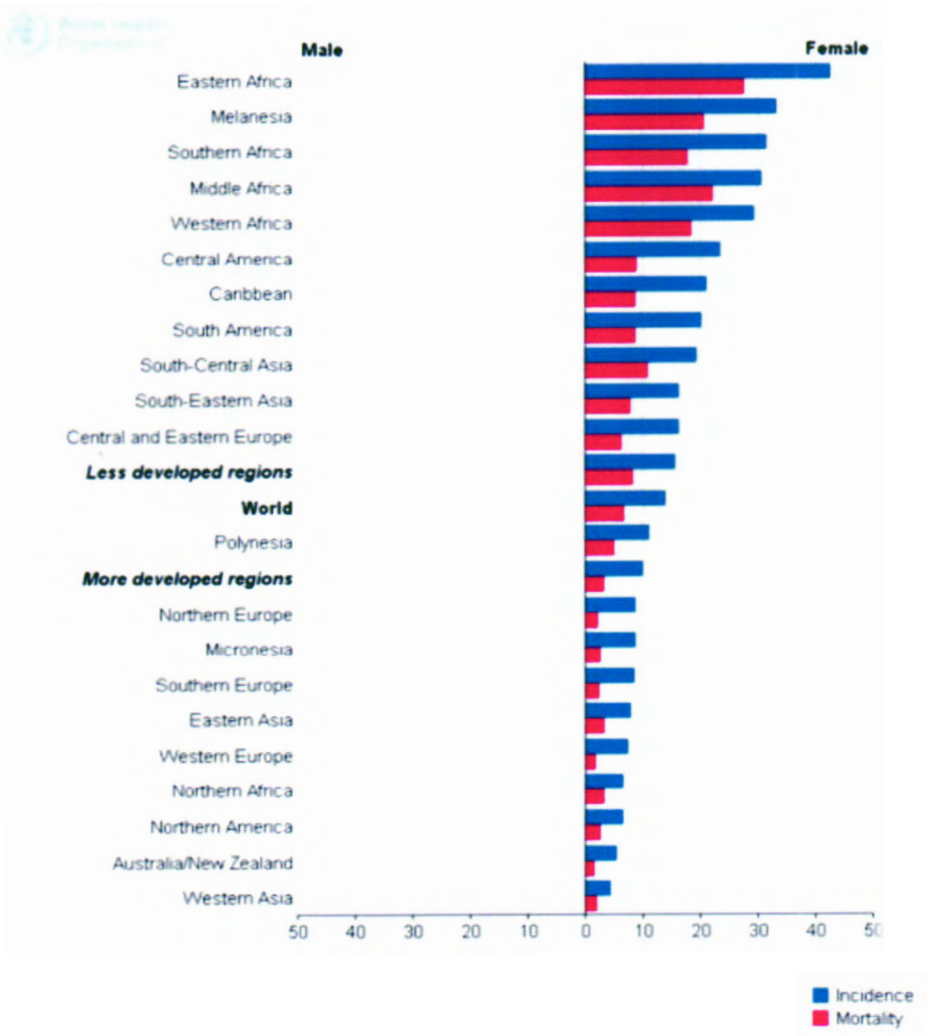


Figure 1.1. Estimated age-standardized rates (World) per 100,000

(GLOBOCAN 2012)

1.2 Prevalence in Asia

In 2012, a meta-static analysis was utilized to explain the pervasiveness and genotype distribution of cervical HPV contamination among FSWs (female sex workers) in Asia. Fourteen qualified studies were distinguished in five databases, and information including 4198 FSWs from nine Asian nations were collected. Rough estimations of cervical HPV predominance among FSWs in this district went from 12.8% to 84.8%. FSWs had an almost 10-fold danger of HPV contamination than the overall public of ladies. Stratified investigation demonstrated that HPV commonness was higher in East Asia than other sub-districts and in more youthful FSWs than older FSWs. HPV genotype transmission was measurably diverse between East Asia and South-east Asia. In East Asia, the most pervasive genotypes were HPV 16 (23.9%), 18 (11.0%), 58 (9.4%), 56 (6.3%) and 52 (5.3%), while they were HPV 52 (12.9%), 16 (8.5%), 58 (5.2%), 18 (5.0%) and 66 (4.9%) in South-east Asia. HPV 31, 33 and 35 were less much of the time found in both sub-districts (Peng et al. 2012).

1.3 Prevalence in Pakistan

In Pakistan, HPV screening is not regularly rehearsed. Furthermore, the transmission mode and risk factors for HPV in Pakistani ladies have not been sufficiently assessed. A noteworthy boundary in building up the study of disease transmission of HPV in this nation is the social forbidden on all matters relating to sex, including sexually transmitted infections (STIs). These socio-social denials make a considerable obstruction to the examination of issues concerning STIs. Subsequently, there are no or next to no information accessible to evaluate the weight of HPV and HPV related cervical carcinoma in Pakistan (Khan et al. 2007; Anwer et al. 1991). Still the scientists are conducting the studies to check out the prevalence of HPV infections. A study revealed 88% and 24% of HPV positive results in cervical cancer women and general population respectively (Raza et al. 2010).

1.4 Human Papillomavirus

There are more than 120 types of HPV distinguished so far described by complete sequencing of genome. HPV can be arranged into two principle phylogenetic genera, the α-HPV and the β- HPV (de Villiers et al. 2004). These compare comprehensively to the mucosal infection causing and cutaneous infection causing HPV separately. The α-HPV incorporate HPV 16, 18, 45, 31 and 33 that bring about 70 percent of instances of cervical disease and are transmitted specifically through sexual contact. Mucosal human papillomavirus can be additionally ordered into low and high risk types relying upon either they cause benign lesions or malignant lesions that might advance to harmful tumors. The β-class cutaneous types, for instance HPV type 5 connected with non-melanoma skin tumor in people who are immunosupperesed or immunocompromised, can be spread straightforwardly through a skin contact. Around 40 human papillomavirus invades the anogenital locale. There are various types that bring about genital warts yet HPV16 and 18 prevail. Anogenital warts infrequently cause injuries that advance to malignancies. Interestingly, the more rich HR anogenital infective types cause intraepithelial neoplasia, or warts, of different anogenital locales, which left uncured can prompt obtrusive tumors, including cervical, vulvar, anal and penile malignancies (Graham. 2010).

Human papillomavirus particles are long circular DNA molecules that consist of 8000 base-pair (bp), wrapped into a protein shell. The genome encodes two proteins called Late proteins (L1 and L2) that make the viral capsids and at least six early proteins (E1, E2, E4–E7) that are necessary for the of the viral DNA replication and assemble the newly produced virus particles within the infected cells (Table.1) (Doorbar et al. 1997). An upstream regulatory region (URR) separates both sets of genes that consist of about 1000 bp. It does not encodes proteins but contains cis-elements required for regulation of gene expression, replication of the genome, and its packaging into virus particles (Fig. 2) (D'Abramo et al. 2011).

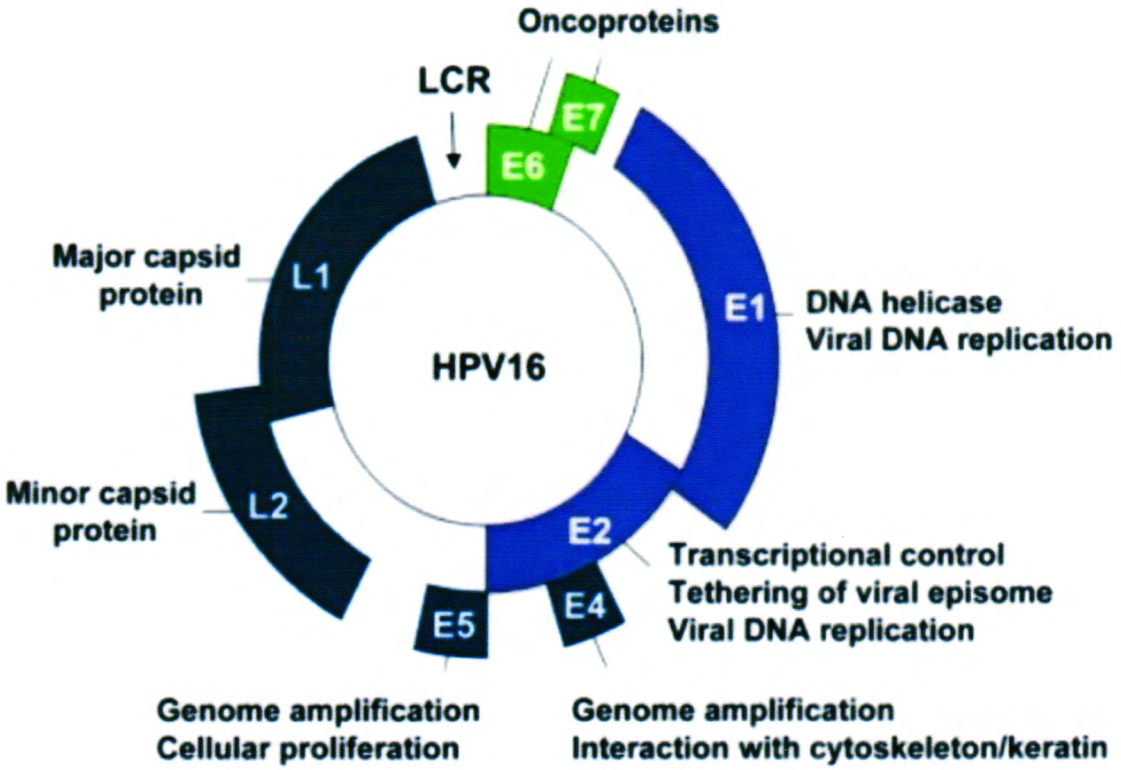


Figure 1.2 Genomic organization of HPV

(D'Abramo et al. 2011)

Table 1.1. Major roles of proteins expressed by high risk HPV (SV Graham, 2010).

Protein	Role in the virus life cycle
E1	Genome replication: ATP-dependent DNA helicase
E2	Genome replication. transcription. segregation. encapsidation Regulation of cellular gene expression Cell cycle and apoptosis regulation
E4	Remodels cytokeratin network. Cell cycle arrest. Virion assembly.
E5	Control of cell growth and differentiation. Immune modulation
E6	Oncoprotein Inhibits apoptosis and differentiation. Regulates cell shape. polarity mobility and signalling
E7	Cell cycle control Controls centrosome duplication.
L1	Major capsid protein
L2	Minor capsid protein Recruits L1 Virus assembly

Human papillomavirus contaminations regularly cause no manifestations, most normal indications of disease are little pink or red warts, tingling and blazing in genital area. Once a female gets to be contaminated with HPV her cervical cells might stay stable and disease might relapse suddenly, or might form into low-grade squamous intraepithelial lesions (LGSILs), additionally termed mild cervical intraepithelial neoplasia (CIN) or early dysplasia. Figure 1.3 gives the summery of HPV disease leading to cervical pre-malignancy and invasive tumor. Low-grade lesions can be prompted by HPV disease. The majority of these lesions either relapse all alone or advancement to high grade squamous intraepithelial lesions (HGSIL) and later into a malignant growth. HGSILs can grow straightforwardly from steady HPV disease or from LGSILs (Outlook, 2000). Some HGSILs will advance to intrusive tumor over a time period of up to 10 years. In this manner, there is abundant time to recognize and cure contaminated females before cervical disease creates. Cervical growth regularly creates in ladies after the age of 40, and the frequency is most elevated among ladies in their 50's and 60's years of age (Parkin et al. 1997).

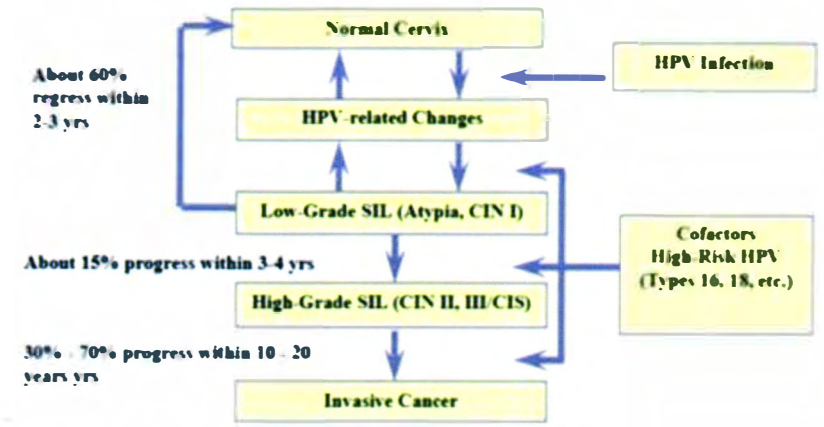


Figure 1.3. Natural history of cervical cancer development

(Blumenthal 2005)

1.5 Introduction of HIV

The Human Immunodeficiency Virus (HIV) which causes the Acquired Immune Deficiency Syndrome (AIDS) was initially found in the mid-1980s. It has spread more quickly than most sicknesses in late history, having social and moral repercussions on people and families. Throughout the years, the connection between HIV/AIDS and poverty has developed and significantly stronger as the disease is contaminating and influencing the more young ones. HIV contaminations are spreading rapidly inside the youth and what transpires today will figure out what is the fate of them and their groups later on. An expected 11.8 million youngsters 15–24 of age are living with HIV/AIDS, and half of every new disease, more than 6,000 day by day, are happening among them (The Joint United Nations Program on HIV/AIDS, UNAIDS, 2003).

HIV/AIDS has been recognized as the world's first health emergency and a critical threat to worldwide population health by The World Health Organization (WHO). According to WHO, it is the second most widely propagating disease and ranked as the 6th common death cause worldwide (WHO, 2004). Recently, on international scale it has been receiving as much concentration as other aspects like war, environmental degradation, terrorism etc. According to the UNAIDS (2006) statistics, AIDS have affected about 65 million people and caused the death of more than 25 million people. It is estimated that by 2020, if prevention and treatment of AIDS is not accelerated the world will be facing 29 million infections.

1.6 HIV and Cervical Cancer

Women infected with HIV are four times more prone to HPV infection and cervical cancer development than non-infected women (Ellerbrock et al. 2000). According to the study, all HPV types were higher in HIV-infected women when compared with non-infected women (Levi et al. 2004). Studies suggest that 20-60 percent of HIV-positive women show signs of pre-cervical cancer. Furthermore, there continues to be a limited understanding of the natural history of HPV and cervical cancer in HIV

infected women, though it is believed that HIV-induced immunosuppression impairs cell-mediated immune control of HPV infections (Palefsky, 2009).

HIV positive women in various geographic regions, for example Africa, seem, by all accounts, to be contaminated with less prevalent types of cancer causing human papillomavirus when contrasted with the remaining regions of the world where HPV types 16 and 18 are more common (McKenzie et al. 2010). Compromised immunity due to HPV/HIV co-infection may prompt systems for LR HPV to cause malignancy in HIV positive females (Chaturvedi et al. 2009).

1.7 Risk Factors

There are many risk factors that can cause cervical cancer. These are: age, multiple sexual partners, parity, exposure to sexually transmitted infections (STIs), oral contraceptives, smoking, use of diethylstilbestrol and immunosuppression due to HIV/AIDS.

1.7.1 HIV/AIDS

HIV contamination quickens the development toward cervical growth and likely is connected with more regrettable clinical outcomes. A current meta-investigation demonstrated that women who have HIV/AIDS have a six-times more danger of cervical malignancy and women who have under-gone organ transplant have more than two-fold the danger of creating cervical tumor, emphatically showing that immune-suppression assumes a part (Grulich et al. 2007). The International Agency for Research on Cancer (IARC) states that HIV is a causal specialist of cervical malignancy (Cogliano et al. 2011).

1.7.2 Age

A solid determinant of the finding of a cancer-causing viral contamination in connection to malignancy progression is appeared to be the age at exposure. The danger of cervical disease is identified with age at first sex. Later studies that included HPV estimations have indicated age at first HPV exposure is really a measure of age at first intercourse (Bosch et al. 2002).

1.7.3 Parity

Females that have had three or more parity are in a like manner supposed to be at higher jeopardy. This is by all accounts because of the distinctive hormonal fluctuations that make them more vulnerable to the human papillomavirus contamination. It is likewise viewed as a high hazard for females at around 17 years or younger when they conceive for the first time. They are twice more at danger of having tumor of cervix later on in life when contrast with the females that get pregnant at the age of 25 a long time and over (American Cancer Society 2010).

1.7.4 Oral contraceptives

The HPV disease cells might be produced quicker through direct cancer-causing activities that are supported by long period of time utilization of contraceptives moreover lead incredibly to the human papillomavirus infection improvement, contraceptives go about as a generative operator with an expansion of 2.2 fold increment in danger level despite the fact that there has been no connection unmistakably clarifying the connection between the HPV infection and contraceptives (Likes et al. 2003).

1.7.5 Sexually Transmitted Infections

Chlamydia trachomatis a microscopic organism is the leading agent for the sexually transmitted bacterial disease that has been discovered to have impact on CIN grade 2. It is an autonomous co-variable to the progress of cervical neoplasia implying that Chlamydia does not bring about the HPV infection or cervical malignancy but rather is a great hazard in supporting its improvement at initial phases of cervical malignancy (Lehtinen et al. 2011).

1.7.6 Smoking

Smoking has been observed to be high hazard element whether it is active or passive. In numerous populations, a solid correlation has been found between smoking propensities

and sexual practices. Cigarettes containing chemical substances have likewise been identified in cervical bodily fluid along these lines therefore helping in improvement of cervical disease by harming DNA around the cervical cells (Özgul, 2009).

1.7.7 Diethylstilbestrol

Diethylstilbestrol (DES) is a medication for hormones for females with high risks of unnatural birth cycle prescribed within the years 1940-1971 has been observed to be of great hazard not to the females taking medications but rather their female offspring. Around 1 out of 1000 of these females create cervical tumor. Those daughters whose mothers used the medicine through the initial 2 months of pregnancy where observed to be of higher danger. The medication still no more being used. Family medicinal history can be a noteworthy danger variable for any female not only those whose mothers utilized the DES hormone prescription additionally ladies whose family history filled with cervical growth (Herbert et al. 2008).

1.7.8 Multiple sex partners

A female having various sexual companions puts her at the higher danger of gaining the human papillomavirus contamination because it is prevailing in men (Likes et al. 2003).

1.8 Screening of cervical cancer

Assessment of the available screening strategies in screening programs have been consistently concentrated on to check screening viability by contrasting the different screening procedures accessible. Almost 266,000 deaths with 85% been from emerging states, death rate increase has been recognized because of the absence of the productive high class pre-cancer screening and treatment assets and also poor framework (Alliance for Cervical Cancer Prevention 2009). Cervical malignancy is well preventable by screening particularly on females that are asymptomatic for precancerous cervical abnormalitites, initial recognition prompts quicker and more effective treatment. As indicated by various studies conducted females that have been screened for any event once in the lifetime between ages 30 and 40 diminish tumor hazard by 25% to 36%

(Cervical Cancer Action 2008).

1.8.1 Pap Smear Screening

Pap smear screening has turned out to be the best strategy for recognizing cervical disease and has facilitated in diminishing illness and death rates achieved by cervical malignancy. Yet around 30% of the outcomes have blunders which happen by means of a consequence of errors like inability of cytotechnologist to identify the existence of irregular cytology or deficient inspection of transformation zone by the doctor (Nouvo et al., 2001).

1.8.2 Liquid Based Cytology (LBC)

Method of collection of sample is like that of the customary Pap smear technique in spite of the fact that an uncommon specimen collecting vial that gathers shed cells from transitioning zone of cervix. The vial contains the mucolytic and haemolytic operators that act as a preservative for the whole apparatus. In this procedure there is even dissemination of cells with diminished cell debris and red blood cells in the sample. This has exceptionally diminished the occurrence of positive incorrect diagnosis of cytology atypia and is better at recognizing squamous anomalies (Kerkar et al., 2006). In spite of its fame there have been no distinctions spotted amongst LBC and Pap smear strategies as far as specificity and affectability, however the straightforwardness in deciphering microscopic readings measures as a benefit with LBC (Hing et al., 2011).

1.8.3 HPV DNA Screening by Southern Blot Hybridization

Different techniques are available for distinguishing HR HPV types which are frequently connected with high grade cervical intraepithelial neoplasia (HGSIL) and obtrusive tumor in the cervix. Methods accessible in this strategy for screening incorporate southern blot hybridation which is said to be a lab best quality level. It is however labourious and tiresome and not extremely appropriate for clinical use since it requests for utilization of fresh cervical tissue which is difficult to direct particularly in mass screening programs (Kerkar et al., 2006).

1.8.4 HPV DNA Screening by Hybrid Capture 2 Assay

A more appropriate method has been utilization of Hybrid capture 2 assay which is generally utilized as a part of HPV DNA screening. Tests for the screening are gotten from cell suspensions gained from LBC or the cytocervical brush utilization (Kerkar et al., 2006).

The study was done to figure out which technique for testing for cervical tumor is better, the ordinary cytology screening strategy or Pap smear screening was thought about against the HPV DNA test screening strategy. In both arms tests were gotten from a VCE smear which comprise of vaginal, cervical and endocervical tests brought with cytobrush from the intersection and endocervix and with two spatulas from ectocervix and vaginal fornices. In the outcomes the HPV DNA screening had more positive readings of any CIN or cervical growth than the traditional cytology screening, unmistakably distinguishing the HPV DNA test to be more delicate particularly in recognizing CIN grade 3 or more. The females found to have positive HPV test results were suggested for concentrated screening (Anttila et al., 2010).

1.9 Vaccination

There are two types of vaccines that have been delivered and endorsed for use. These antibodies are Gardasil which was endorsed by food and drugs administration (FDA) delivered by Merixk (white house station, NJ), the other one was Cervarix created by Glaxo Smithkilne (Philadelphia, PA) and is presently affirmed in Australia and Europe with ceaseless assessment under the FDA (Godrey, 2007) The immunizations are both managed as prophylactic antibodies which implies they are uniquely made for counteracting against conceivable HPV contaminations instead of going about as restorative antibodies which treat gained diseases. For a man to be all around secured they must be directed with the three measurements arrangement. Immature young ladies that have been vaccinated ought to likewise go to screening programs when they are of age following the antibodies can't secure around 30% of the HPV sorts along these lines the need to screen their status (Jemal et al. 2011).

1.10 Objectives

The aims of study are:

- To assess the prevalence of HPV in HIV positive female patients in Islamabad (Pakistan)
- To evaluate the risk factors associated with HPV
- Molecular detection of HPV in HIV positive samples using PCR
- Genotyping of selected samples using restriction fragment length polymorphism (RFLP)
- To find the association of HPV infection in HIV positive patients.

Chapter

2. Materials and Methods

2.1 Sample Collection

This study was approved by the Board of Ethical Committee of International Islamic University and samples were obtained by the informed consent of the patient. About 50 HIV positive women participants from were interviewed using a questionnaire to obtain the following information: demographic characteristics, sexual behavior, contraceptive use, drug and tobacco use, and history of gynecological and sexually transmitted diseases. Specimens were collected from the endocervical canal using Cytobrush by the assistance of gynecologists working in PIMS hospital of Islamabad.

2.2 DNA Extraction

DNA was extracted in the Biosafety level 2 lab of NORI hospital, Islamabad. Using phenol chloroform method 3 different solutions A, B and C were prepared in different molar concentration (***Appendix I***). According to the protocol following steps were followed:

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 500µl of solution A.
3. Solution was kept at the room temperature for 8-10 minutes.
4. The mixture was then centrifuged at 13000 rpm for 1-3 minutes and the supernatant was separated and discarded.
5. The pellet obtained and re-suspended in 400 µl of solution A and mixed by inverting several times.
6. The mixture was again centrifuged and the supernatant was separated and discarded.
7. Then 400 µl of solution B was added.
8. For the degradation of impurities, 12µl of 20% of SDS and 25µl of protenase K was added and incubated at 37°C.

9. 0.5 ml of solution C and equal amount of phenol was added after incubation and inverted several times.
10. Then it was centrifuged at 14000rpm for 8-10 minutes.
11. The upper layer was transferred into new eppendorf tube and equal amount of solution C and phenol was added. Again centrifuged the mixture at 14000rpm for 8-10 minutes.
12. The aqueous phase was collected in a separate eppendorf tube.
13. DNA was precipitated by adding 55µl of sodium acetate and 1000µl of 100% chilled ethanol and tube was inverted several times.
14. Then DNA was settled by centrifuging the tube at 13000 rpm for 1 minute and supernatant was separated and discarded.
15. 0.5ml of 70% ethanol was added to wash the DNA pellet.
16. Supernatant was discarded and air dried at room temperature by inverting the tubes.
17. The precipitated DNA was dissolved in 60µl of TE buffer (10mM Tris, 0.1mM EDTA) and stored at room temperature.

2.3 Primers

The DNA extracted was then analyzed for the presence of HPV DNA sequences, using a set of degenerate primers F1 and R1, for targeting 450bp fragment of L1 Open Reading Frame (ORF) (Manos et al., 1989; Bernard et al., 1994; Cappiello et al., 1997; Chan et al., 2001). Table 2.1 represents the sequences of the primers.

Table 2.1 Sequence of primers.

Primers	*Sequence*
F1	**CGTCCACAAGAGGGATACTGATC**
R1	**GCACCAGGGATCATAACTAATGG**

Shikova et al., 2009.

Table 2.2. PCR conditions for F1/R1 primers

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

2.4 Polymerase Chain Reaction Amplification

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 µl volume. Each reaction contained

DNA sample	2 µl
10 x buffer	5 µl
dNTPs	200 µM
MgCl2	3 µM,
β-globin primer	0.1 µM
Taq polymerase	1.25 units

The PCR protocol uses 40 cycles with the conditions shown in table 2.2 (Randolph *et al.* 2000). 2% agarose gel stained with ethidium bromide was used to analyze PCR products. It was visualized and photographed on a Gel Doc (Bio Rad).

2.5 Gel Electrophoresis

The obtained amplified product from both PCR procedures was analyzed by using 2% agarose gel electrophoresis technique. Gel was prepared by adding 2g of agarose in 100ml of 1X TBE buffer solution. A homogenized solution was obtained by heating the solution. 30µl of Ethidium Bromide was added for visualization. After solidification of gel, product and marker of 50bp was used for the comparison.

2.6 Restriction Fragment Length Polymorphism (RFLP)

RFLP was used to analyze the positive amplified samples of L1 consensus region of HPV genome by using F1/R1 (Table 2.1). This helped in the detection of the HPV types present in it. The PCR product was digested by NlaIII enzyme. In a 20-µl mixture, 6 µl of positive amplified PCR products, 1 U of NlaIII, 2 µg of bovine serum albumin (BSA) and 2 µl of 10_ NE Buffer 4 were mixed, and the solution was incubated at 37°C for 16 hr.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

An 8% polyacrylamide gel (***Appendix II***) was used for loading and visualization of the digested products (10 µl). 1x TBE buffer was prepared by diluting 10x TBE buffer in 1:9 ratio (***Appendix III***) for the gel tank. The gel was stained with 0.5 mg/ml Ethidium Bromide after electrophoresis and the fragments were visualized using a Bio-Rad Gel Doc system. Theoretical digestion patterns were obtained using the NEB cutter V2.0 tool. GenBank Database was utilized to obtain the DNA sequence of consensus L1 region of HPV.

2.8 Sequencing

HPV DNA was used for the sequence purpose. For this Gene JET^{TM} Gel Extraction Kit was used to elute the DNA of the amplified product from the gel.

2.9 Statistical Analysis

IBM SPSS version 24 system was used for the statistical analysis of the results obtained.

Chapter

3. Results

50 HIV positive patients from special clinic of HIV in Pakistan Institute of Medical Sciences (PIMS) Islamabad were selected to carry out this study. These samples were selected on the basis of age group, parity, contraception and marital status. The sample was collected with the consent of the patient. These results are analysed on the basis of the frequencies, standard deviations percentages and mean by using different tabulation methods and graphical representations.

3.1 Screening of HPV DNA

F1/R1 primer set (table 2) was used to amplify the 450bp fragment of HPV DNA by using PCR. Reaction conditions are mentioned in the methodology section. The amplified samples were run on the 2% agarose gel. Figure 3.1 shows the results of the PCR amplification. Fragment of 450bp in an amplified product shows the presence of HPV DNA. Out of 65 samples that were tested for Human Papillomavirus, 30 samples showed bands after PCR amplification. Thus, prevalence of HPV in the selected population was found to be 30/50 (60%).

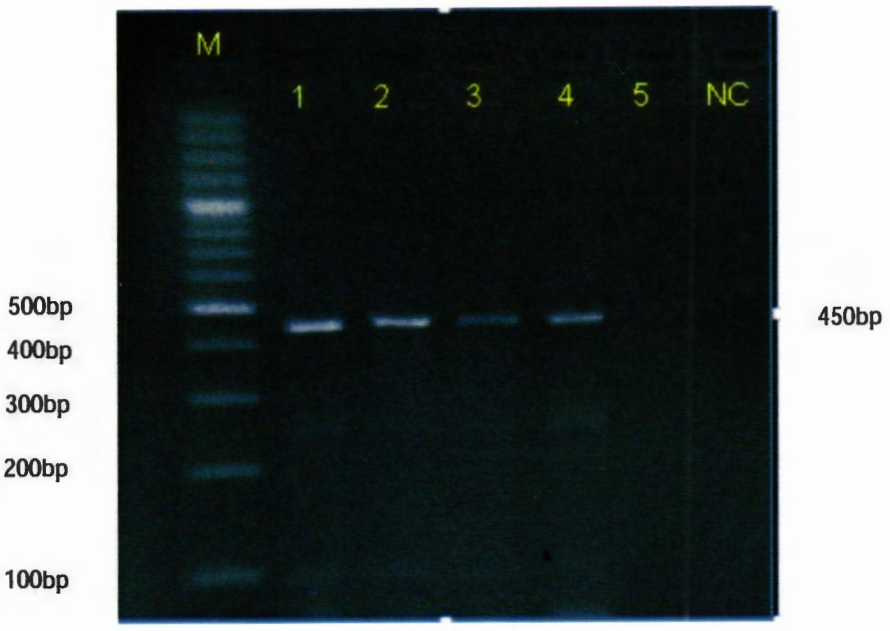


Figure 3.1. Amplified product by using F1/R1 primers on 2% Agarose gel.

Lane M: 100bp ladder

Lane 1-4: positive HPV samples with band size 450bp

Lane 5: negative sample

Lane 6: negative control (NC).

3.2 Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP was used to detect the HPV genotypes in the amplified positive products. These amplified products were digested with NlaIII enzyme. After 16 hours of incubation, the samples were run on 8% polyacrylamide gel electrophoresis (PAGE) for the visualization of the restriction patterns. These restriction patterns were analysed by using NEB cutter, which shows different band patterns for different HPV genotypes (HPV 16, HPV 18, HPV 45 and HPV 33)(figure 3.2, 3.3, 3.4, 3.5). Figure 3.2 shows the result of restriction patterns on PAGE.

Results obtained showed the presence of different High risk and Low risk types of HPV. Out of 30 HPV positive samples, 9 (18%) were found to be high risk HPV 18, 5 (10%) were found to be high risk HPV 16, 4 (8%) were HPV type 33 and 2 (4%) were HPV type 45. The remaining 11 (22%) positive samples were low risk HPV subtypes. These results showed that among High risk types HPV type 18 was most prevalent i.e. 18%. While prevalence of HPV 16 is 10%, HPV 33 is 8% and HPV type 45 was 4% prevalent. The P value of HPV 18 was P=0.03, HPV 16 P= 0.0264, HPV 45 P=0.0184 and HPV 33 P=0.042, which means it was less than 0.05. These results showed the significance of HPV genotypes.

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

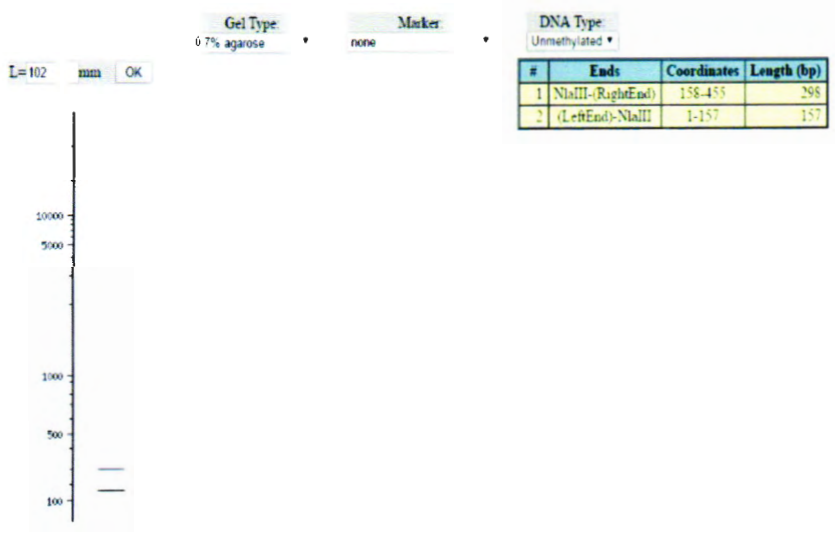
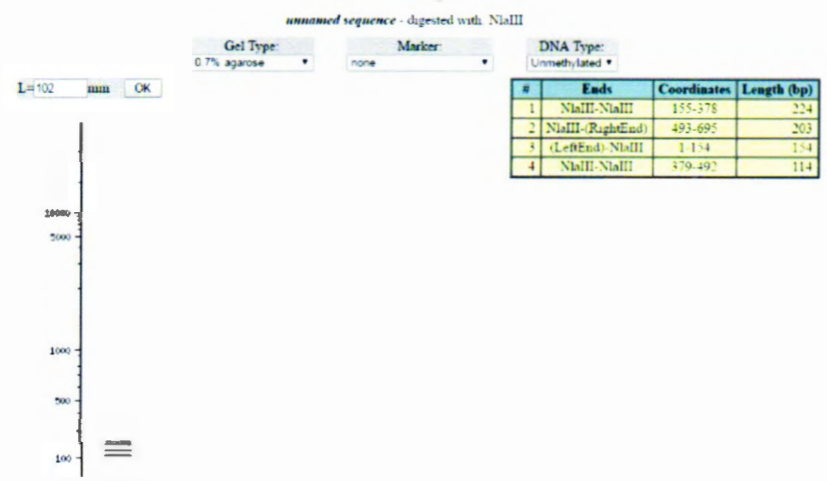


Figure 3.3. NEB Cutter tool gel image for HPV type 45



#	Ends	Coordinates	Length (bp)
1	NlaIII-NlaIII	155-378	224
2	NlaIII-(RightEnd)	493-695	203
3	(LeftEnd)-NlaIII	1-134	134
4	NlaIII-NlaIII	379-492	114

Figure 3.4. NEB Cutter tool gel image for HPV type 18

#	Ends	Coordinates	Length (bp)
1	NlaIII-(RightEnd)	438-696	259
2	NlaIII-NlaIII	202-375	174
3	NlaIII-NlaIII	60-201	142
4	NlaIII-NlaIII	376-437	62
5	(LeftEnd)-NlaIII	1-59	59

Figure 3.5. NEB Cutter tool gel image for HPV type 33

Figure 3.6. RFLP using NlaIII enzyme

Lane 1R shows the restricted fragments of HPV type 16 at around 420 and 280 bp

Lane 2R shows the restricted fragments of HPV type 45 at around 280 and 157 bp

Lane 3R shows the restricted fragments of HPV type 18 at around 224, 203, 154 and 114 bp

Lane 2R shows the restricted fragments of HPV type 33 at around 259, 174 and 143bp

Lane 1, 2, 3 and 4 shows the uncut product whereas NC and NCR are the uncut product and restricted product of negative control respectively.

3.3 STATISTICAL ANALYSIS

50 patients that belong to different age groups and marital statuses were taken for the analysis. Out of 50, 2 of the patients were unmarried, 15 were widows and rest were married. Their full term pregnancies status was also different. The other clinical and physical parameters of 30 positive HPV patients is shown in table 3.1. These parameters are the gynaecological issues that usually a woman suffer from. The mean and standard deviation of these variables were analysed. Table 3.2 shows the mean value and standard deviation.

Table 3.1 Clinical and physical parameters of HIV patients positive for HPV

Parameters		Percentages
Vaginal discharge	Yes	63.3%
	No	36.7%
Fever	Yes	33.3%
	No	66.7%
Abdominal pain	Yes	36.7%

Post coital bleeding	No	63.3%
	Yes	46.6%
Dyspareunia	No	53.3%
	Yes	55%
Contraception	No	45%
	Yes	86.7%
	No	13.3%

Table 3.2 Standard deviation and mean values of different variables

Variable	*N*	*Minimum*	*Maximum*	*Mean*	*Std. Deviation*
Age	30	22	58	38.33	±9.94
Marital years	30	1.00	5.00	3.2000	±1.0635
Parity	30	1.00	3.00	1.7667	±0.6789

3.3.1 HPV infection related to age of HIV patients

All of the patients under study were older than 20 years of age. Out of 30 positive patients, 15 (50%) lied between the group of 21-30 years, 10 (33.3%) lied between the group of 31-40 years, 4 (13.3%) lied in the group of 41-50 years and only 1(3.3%) patient was older than 51 years. Age group of 21-30 years showed the highest percentage of HPV infection (Figure 3.7). Tabular form of the results is shown in table 3.3. The standard deviation evaluated was ±9.94 given in table 3.2.

Table 3.3. Frequency and percentage of HPV positive patients of different age groups

Age	***Frequency***	***Percentage***
20 years and below	None	None
21-30 years	15	50%
31-40 years	10	33.3%
41-50 years	4	13.3%
51 years and above	1	3.3%

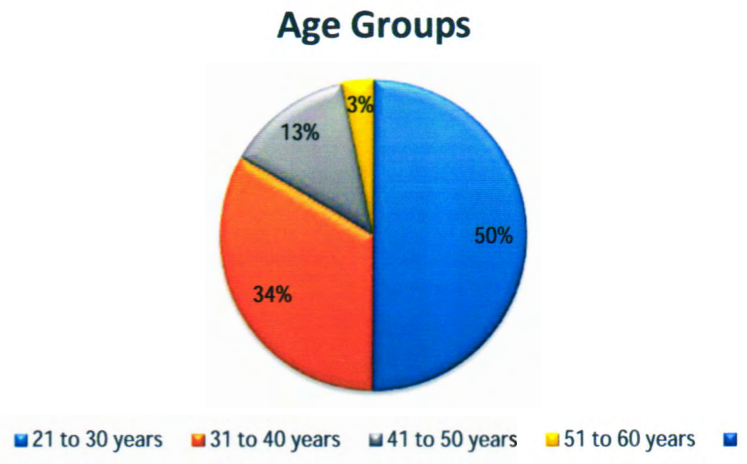


Figure 3.7. Age distribution of HIV patients positive for HPV infection

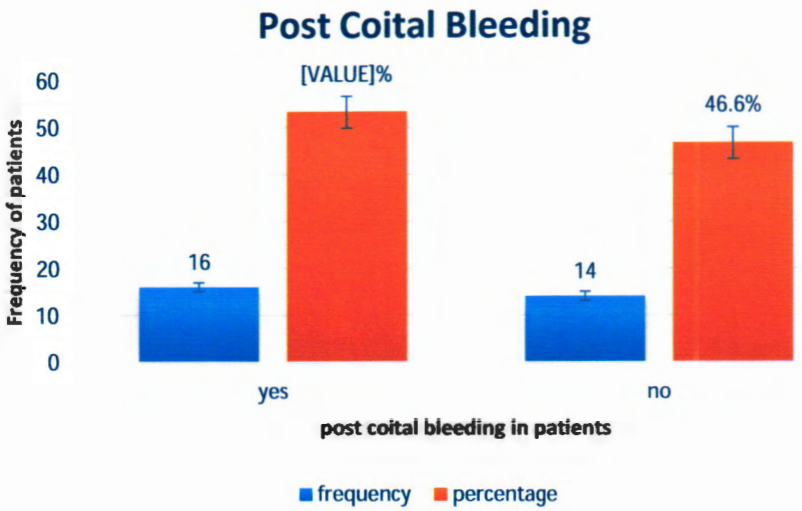


Figure 3.11. Post coital bleeding frequencies and percentages in HIV patients positive for HPV

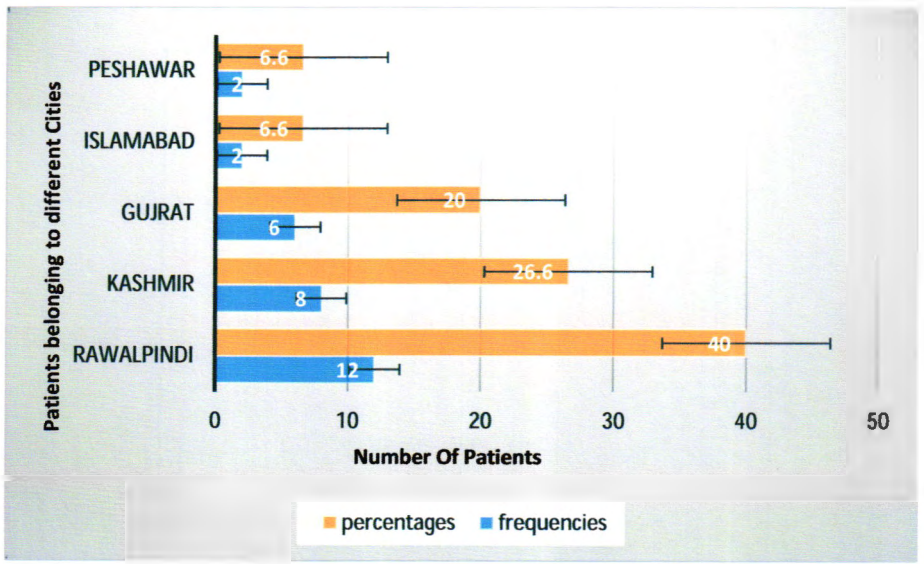


Figure 3.12. HPV infection incidence in patients belonging to different cities of Pakistan

3.3.7 HPV infection related to the educational status of HIV patients

Most of the HIV patients were less educated. Out of 30 HPV positive patients, 17 (56.6%) had no education at all. While 5 (16.6%) had passed matric, 2 (6.67%) had passed 8^{th} grade, 4 (13.33%) had passed 5^{th} grade and only 2 (6.67%) were graduates (Figure 3.13).

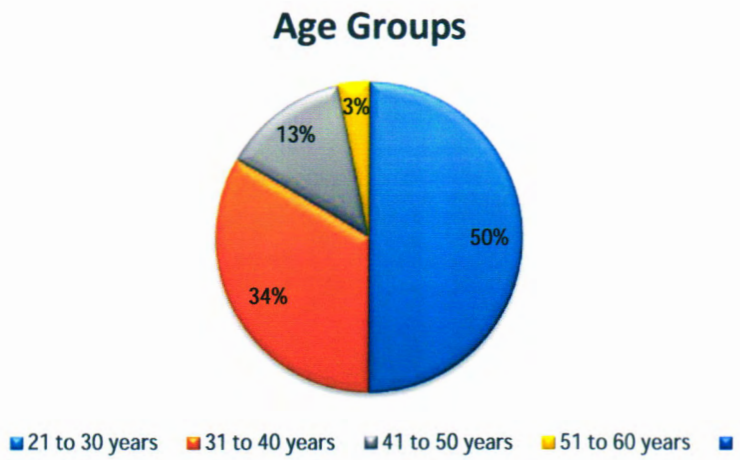


Figure 3.7. Age distribution of HIV patients positive for HPV infection

3.3.2. HPV infection related to marital years of HIV patients

2 (4%) out of 50 patients were unmarried, 15 (30%) were widows and 33 (66%) were married. Among the total 30 HIV patients that are positive for HPV, 8 (26.6%) were married for 1 to 10 years, 14 (46.6%) were married for 11-20 years, 6 (20%) for 21-30 years, 1(3.33%) were married for 31-40 years and 1(3.33%) were married 41-50 years (Figure 3.8, Table 3.4).

Table 3.4. Frequency and percentage of HPV positive patients' years married for

Marital years group	***Frequency***	***Percentage***
Less than 1 years	None	None
1-10 years	8	26.6%
11-20 years	14	46.6%
21-30 years	6	20%
31-40 years	1	3.33%
41-50 years	1	3.33%
More than 50 years	None	None

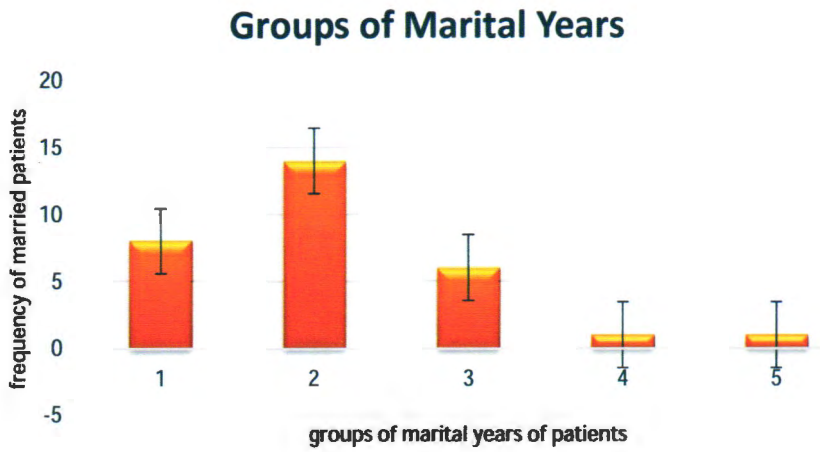


Figure 3.8. Marital years of HIV patients positive for HPV

Bar1: 1 to 10 years, Bar 2: 11 to 20 years, Bar 3: 21 to 30 years, Bar 4: 31 to 40 years and Bar 5: 41 to 50 years.

3.3.3. HPV infection related to the full time pregnancy (Parity) in HIV patients

Out of 30 HPV positive patients, 7 (23.3%) patients were those who had completed 2 full term pregnancy, 9 (30.0%) patients had undergone 3 full term pregnancies, 6 (20%) patients completed 4 full time pregnancies, 5 (16.6%) patients completed 5 full time pregnancies while 3(10%) patients completed 8 full term pregnancies. The trend followed by the given results is given in Figure 3.9. Table 3.5 shows the frequencies and percentages of the positive patents.

Table 3.5. Frequencies and percentages of parity of HIV patients positive for HPV

Parity	*Frequency*	*Percentage*
2	7	23.3%
3	9	30%
4	6	20%
5	5	16.6%
8	3	10%

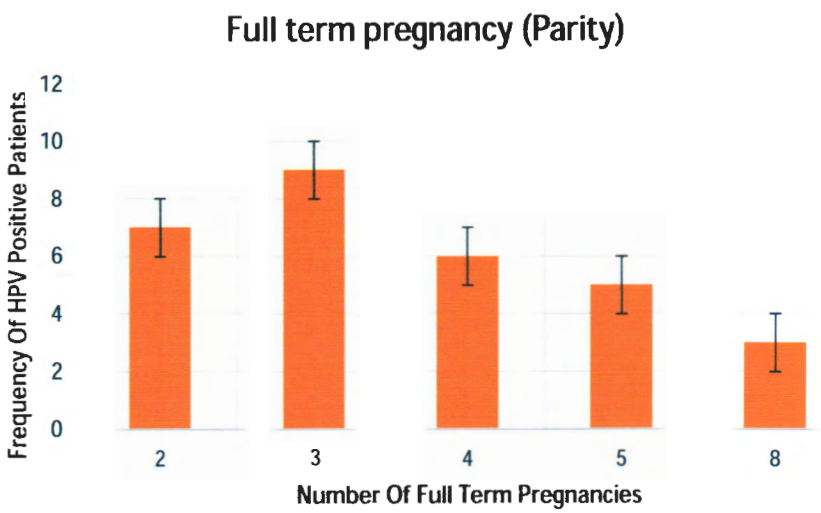


Figure 3.9. Parity of HIV patients positive for HPV

3.3.4. HPV infection related to the methods of Contraception in HIV patients

Out of 30 positive patients, 10 (33.3%) had used barrier as a mean for contraception, while 8 (26.6%) used oral contraceptives, 4 (13.3%) used implinon and 3 (10%) were using intra-uterine contraceptive devices. 5 (16.6%) patients haven't used any means of contraception (Figure 3.10, table 3.6).

Table 3.6 Frequencies and percentages of contraception modes of HIV patients positive for HPV

Contraception modes	***Frequency***	***Percentage***
Barrier	10	33.3%
Oral	8	26.8%
Iucd	3	10%
Implinon	4	13.3%
None	5	16.6%

3.3.5. HPV infection related to post coital bleeding in HIV patients

Among the 30 HPV positive patients, 16 (53.3%) complained about post coital pain while rest of the 14 (46.6%) patients didn't complain. Figure 3.11 reveals the results.

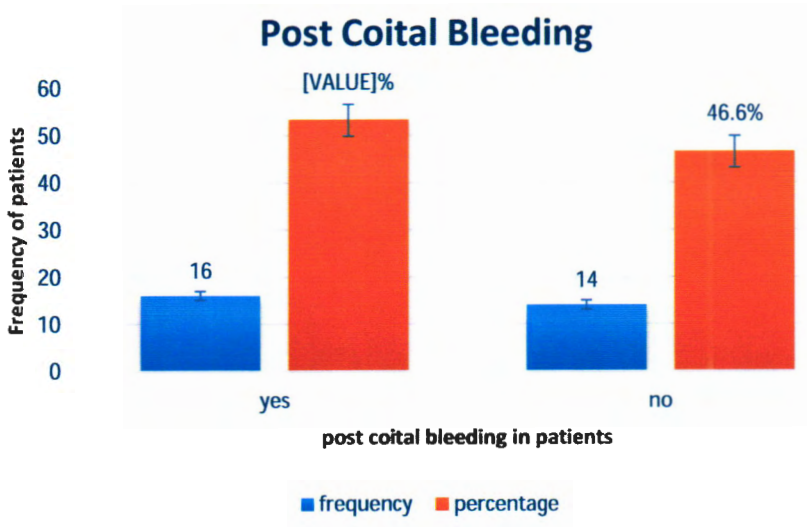


Figure 3.11. Post coital bleeding frequencies and percentages in HIV patients positive for HPV

3.3.6 HPV infection incidence in different areas

Among the 30 HPV positive patients, 12 (40%) were the resident of Rawalpindi, 8 (26.6%) were from Kashmir and 6 (20%) were residents Gujrat, 2 (6.6%) were from Islamabad and 2 (6.6%) from Peshawar cities (Figure 3.12).

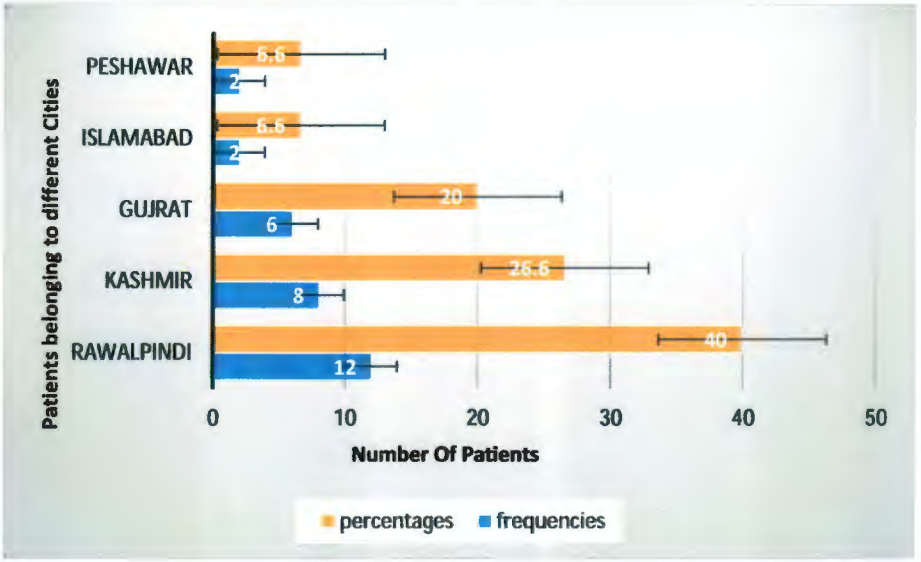


Figure 3.12. HPV infection incidence in patients belonging to different cities of Pakistan

3.3.7 HPV infection related to the educational status of HIV patients

Most of the HIV patients were less educated. Out of 30 HPV positive patients, 17 (56.6%) had no education at all. While 5 (16.6%) had passed matric, 2 (6.67%) had passed 8^{th} grade, 4 (13.33%) had passed 5^{th} grade and only 2 (6.67%) were graduates (Figure 3.13).

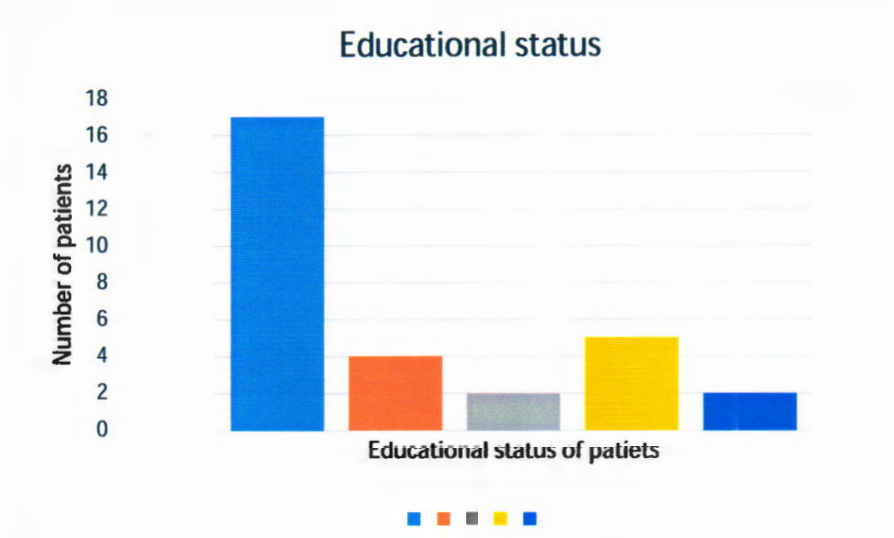


Figure 3.13. Educational status of HIV patients positive for HIV infection

3.4 Elution of DNA from the Gel for DNA sequencing

Gene JET^{TM} Gel Extraction Kit was used to elute the DNA of the amplified product from the gel by using the manufacturer's protocol. The required band pattern was obtained by running the amplified product on 2% agarose gel. A sterile sharp blade was used to excise the bands from the gel 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.

3.5 Phylogenetic Analysis:

A phylogenetic analysis was done by using Neighbour Joining method for the results we obtained. In this study four different HPV gene sequences of HPV are reported. Fifteen sequence of HPV genotype 16, 18, 45, 11, 6 and 33 were obtained from NCBI. All these sequences were used to construct a phylogenetic tree as shown in figure 3.14. All sequence is grouped into five clusters. According to the Phylogenetic analysis of HPV sequences NORI 18 sequence was connected with other four sequences of HPV 18 reported worldwide and grouped in cluster 1. Similarly, HPV 16 lie in cluster 2 where it was linked with other HPV sequences from different areas of the world. 2 sequences of NORI HPV 45 have shown close association and found in separate cluster 3 but one of the NORI HPV Type 45 showed similarity with other sequences of HPV Type 45 of world hence grouped in cluster 4. Sequence of HPV 33, HPV 11 and HPV 6 were closely related and grouped in cluster 5.

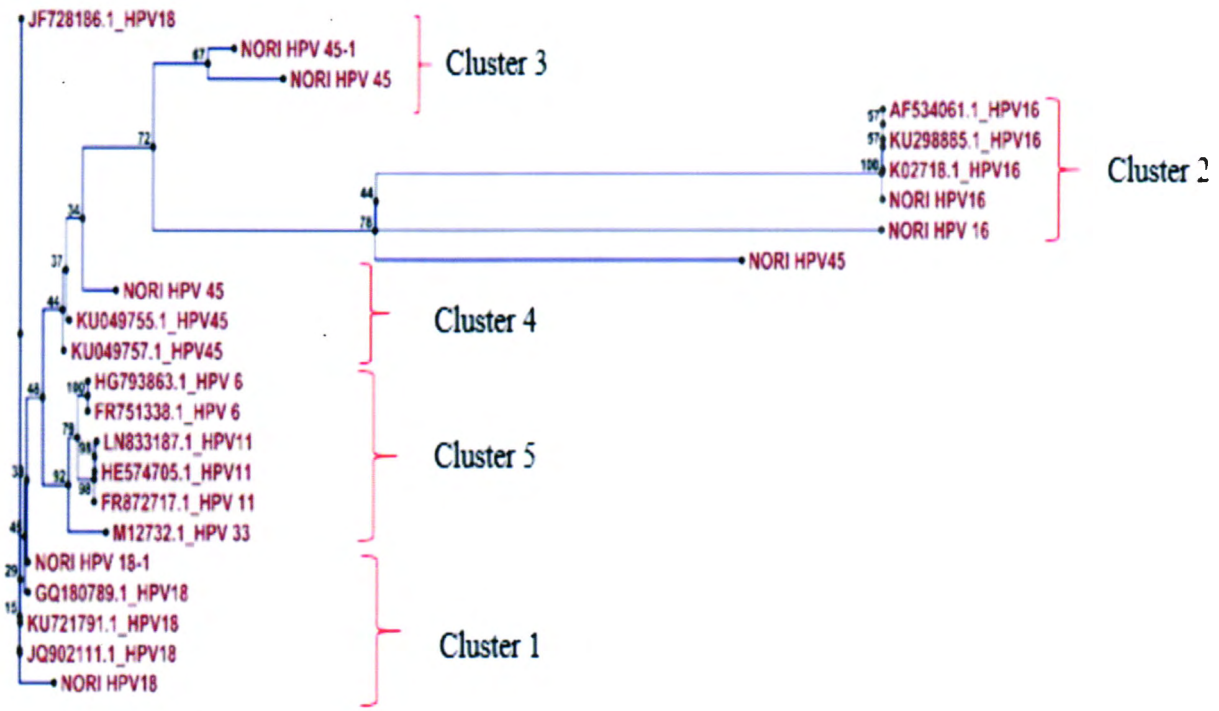


Figure 3.14 Phylogenetic Tree of HPV sequence by using Neighbour Joining method

5. References

Alliance for cervical cancer prevention. (2009). New evidence on the impact of cervical cancer screening and treatment using HPV DNA tests,visual inspection, or cytology; Cervical cancer prevention fact sheet.

American Cancer Society. 2010, 1-9. Cervical cancer.

Anttila A, Katoniemi-Talonen L, Lainonen M, Hakama M, Laurila P, Tarkkanen J, Maila N, Nieminen P. (2010). Rate of cervical cancer, severe intraepithelial neoplasia and Adenocarcinoma in situ in primary HPV DNA screening with cytology triage; randomized study within organized program. *BMJ journal*. 340: 1804.

Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM.(1994). Identification and Assessment of Known and Novel Human Papillomaviruses by Polymerase Chain Reaction, Restriction-Fragment-Length Polymorphisms, Nucleotide Sequence, and Phylogenetic Algorithms. *J. Infect. Dis.*170:1077–1085.

BoschF X, LorinczA, MuñozN, MeijerC J L M, ShahK V. (2002). The causal relation between human papillomavirus and cervical cancer. *J ClinPathol.* 55:244-26.

C.M. D'Abramo and J. Archambault. (2011). Small Molecule Inhibitors of Human Papillomavirus Protein - Protein Interactions. *The Open Virology Journal*. 5:80-95.

Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, Daniel R, Shah KV, Sidransky D. (2000). Detection and Quantitation of Human Papillomavirus (HPV) DNA in the Sera of Patients with HPV-associated Head and Neck Squamous Cell Carcinoma. *Clinical Cancer Research*.6: 4171–4175.

Cappiello G, Garbuglia AR, Salvi R, Rezza G, Giuliani M, Pezzotti P, Suligoi B, Branca M, Migliore G, FormigoniPomponi D, D'Ubaldo C, Ippolito G, Giacomini G, Benedetto A.(1997). HIV Infection Increases the Risk Of Squamous Intra-EpithelialLesions In

Women With HPV Infection: An Analysis Of HPV Genotypes. *Int. J. Cancer.*72:982–986.

Castle PE, Walker JL, Schiffman M, Wheeler CM.(2005). Hormonal contraceptive use, pregnancy and parity, and the risk of cervical intraepithelial neoplasia 3 among oncogenic HPV DNA-positive women withequivocal or mildly abnormal cytology. *Int J Cancer.* 117(6):1007-12.

Chan PK, Chan DP, To KF, Yu MY, Cheung JL, Cheng AF.(2001). Evaluation of extraction methods from paraffin wax embedded tissues for PCR amplification of human and viral DNA. *J ClinPathol.*54:401–403.

Chaturvedi A, Madeleine M, Biggar R, Engels E. (2009). Risk of human papillomavirus-associatedcancers among persons with AIDS. *J Natl Cancer Inst.* 101:1120 - 1130.

Cogliano VJ, Baan R, Straif K, Grosse Y, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-TallaaL, Guha N, Freeman C, Galichet L, Wild CP. (2011). Preventable exposures associated with human cancers. *JNCI.* 103: 1827-39.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zurHausen H. (2004). Classification of papillomaviruses. *Virology.* 324: 17–27.

Doorbar J, Foo C, Coleman N, Medcalf L, Hartley O, Prospero T, Napthine S, Sterling J, Winter G, Griffin H. (1997). Characterization of events during the late stages of HPV16 infection *in vivo* using high-affinity synthetic Fabs to E4. *Virol.* 238:40–52.

Ellerbrock TV, Chiasson MA, Bush TJ, Sun XW, Sawo D, Brudney K, Wright TC Jr. (2000). Incidence of cervical squamous intraepithelial lesions in HIV infected women. *JAMA.* 283:1031.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F.(2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 136: 359-386.

Gary M. Clifford, Maria Alice G, Gonc¸ Silvia Franceschi. (2006). Human

papillomavirus types among women infected with HIV: a meta-analysis. *AIDS*. 20:2337–2344.

GLOBOCAN 2012 (IARC), Section of Cancer Surveillance (23/4/2016)

Godfrey J. (2007). Towards optimal Health, Diane M. Harper, M.D., M.S., M.P.H. Discusses the HPV vaccine and prevention of cervical cancer. *Journal of women's Health*. 16(10):139-1401.

Groesbeck P. Parham, Vikrant V. Sahasrabuddhe, Mulindi H. Mwanahamuntu, Bryan E. Shepherd, Michael L. Hicks, Elizabeth M. Stringer, Sten H. Vermund. (2006). Prevalence and predictors of squamous intraepithelial lesions of the cervix in HIV-infected women in Lusaka, Zambia.*Gynecologic Oncology*. 103:1017–1022.

Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. (2007). Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*. 370(9581):59-67.

Herbert J, Janis C. (2008). Reducing patient risk for human papillomavirus infection and cervical cancer. *Journal of the American osteopathic association*. 108 (2): 65-70.

Hildesheim A, Herrero R, Castle PE, Wacholder S, Bratti MC, Sherman ME, Lorincz AT, Burk RD, Morales J, Rodriguez AC, Helgesen K, Alfaro M, Hutchinson M, Balmaceda I, Greenberg M, Schiffman M. (2001). HPV co-factors related to the development of cervical cancer: results from a population-based study in Costa Rica. *Br J Cancer*. 84(9):1219-26.

Hing E, Saraiya M, Roland KB. (2011). Liquid-based Cytology Test use by office-based physicians.United States, 2006-2007. *National Health Statistics*. 40: 2-8.

Holmes KK, Levine R, Weaver M. (2004). Effectiveness of condoms in preventing sexual transmitted infections. *Bull World Health Organ*. 82(6):454-61.

Jaya Chakravarty, AnkitaChourasia, Minaxi Thakur, Abhishek Kumar Singh, Nisha Rani Agrawal, ShyamSundar. (2016). Prevalence of human papillomavirus infection &

P.D. Blumenthal, M. Lauterbach, J.W. Sellors, R. Sankaranarayanan.(2005). Training manual "cervical cancer prevention for low resource setting". JHPIEGO- an affiliate of Johns Hopkins University

Palefsky J. (2009). Human papillomavirus-related disease in people with HIV.*CurrOpin HIV AIDS.* 4(1):52–56.

Parkin DM, Bray F. (2006). Chapter 2: the burden of HPV-related cancers. *Vaccine.* 24(Suppl 3):S3/11–25. 5.

Parkin DM, Iscovich J.(1997). Risk of cancer in migrants and their descendants in Israel: II. Carcinomas and germ-cell tumors. *Int J Cancer.*70(6): 654-60.

Peng RR, Li HM, Chang H, Li JH, Wang AL, Chen XS. (2012). Prevalence and genotype distribution of cervical human papillomavirus infection among female sex workers in Asia: a systematic literature review and meta-analysis. *Sex Health* . 9(2):113-9.

Rachel H Gormley, Carrie L Kovarik. (2009). Dermatologic manifestations of HPV in HIV-infected individuals. *Current HIV/AIDS Reports.* 6:130–138.

SA Raza, S Franceschi, S Pallardy, FR Malik, BI Avan, A Zafar, SH Ali, S Pervez, S Serajuddaula, PJF Snijders, FJ van Kemenade, CJLM Meijer, S Shershah and GM Clifford. (2010). Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. *British Journal of Cancer.*102: 1657 – 1660.

Saeed Khan, Nadeem N. Jaffer, Mohammad Nasherwan Khan, Mohammad A. Rai, Majid Shafiq, Adnan Ali, Shahid Pervez, Nusrat Khan, Aliya Aziz, Syed H. Ali. (2007). Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma. *International Journal of Infectious Diseases.* 11: 313—317.

Susan Hariri, Elizabeth R. Unger, Maya Sternberg, Eileen F. Dunne, David Swan, Sonya Patel, and Lauri E. Markowitz. (2011). Prevalence of Genital Human Papillomavirus among Females in the United States, the National Health and Nutrition Examination Survey, 2003– 2006. *JID.*204.

SV Graham. (2010). Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies.*Future Microbiol.* 5(10): 1493–1506.

UNAIDS 2003. HIV/AIDS and Young People: Hope for tomorrow. Viewed 15.11.2007. http://data.unaids.org/publications/IRC-pub06/jc785.

UNAIDS 2006. Global Facts and Figures, viewed 18- November 2007. http://data.unaids.org/pub/GlobalReport/2006/200605 FS_globalfactsfigures_en.pdf.

WHO 2004. The Global Burden of Disease. Viewed 24 .02.2009. http://www.who.int/healthinfo/global_burden_disease/GBD_report_20 04update_full.pdf.

Woodman CB, Collins SI, Young LS. (2007). The natural history of cervical HPV infection: unresolved issues. *Nat Rev Cancer.*7: 11-22.

Zahid B, Khawaja N, Tayyeb R. (2005). Prevalence of abnormal cervical cytology and its relation with age and parity. *Ann King Edward Med Coll.* 11 (4): 524-5.

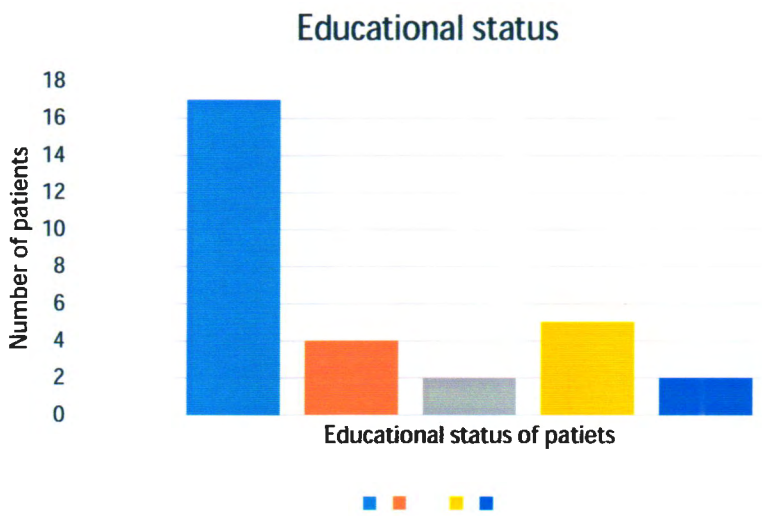


Figure 3.13. Educational status of HIV patients positive for HIV infection

3.4 Elution of DNA from the Gel for DNA sequencing

Gene JET^{TM} Gel Extraction Kit was used to elute the DNA of the amplified product from the gel by using the manufacturer's protocol. The required band pattern was obtained by running the amplified product on 2% agarose gel. A sterile sharp blade was used to excise the bands from the gel 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.

3.5 Phylogenetic Analysis:

A phylogenetic analysis was done by using Neighbour Joining method for the results we obtained. In this study four different HPV gene sequences of HPV are reported. Fifteen sequence of HPV genotype 16, 18, 45, 11, 6 and 33 were obtained from NCBI. All these sequences were used to construct a phylogenetic tree as shown in figure 3.14. All sequence is grouped into five clusters. According to the Phylogenetic analysis of HPV sequences NORI 18 sequence was connected with other four sequences of HPV 18 reported worldwide and grouped in cluster 1. Similarly, HPV 16 lie in cluster 2 where it was linked with other HPV sequences from different areas of the world. 2 sequences of NORI HPV 45 have shown close association and found in separate cluster 3 but one of the NORI HPV Type 45 showed similarity with other sequences of HPV Type 45 of world hence grouped in cluster 4. Sequence of HPV 33, HPV 11 and HPV 6 were closely related and grouped in cluster 5.

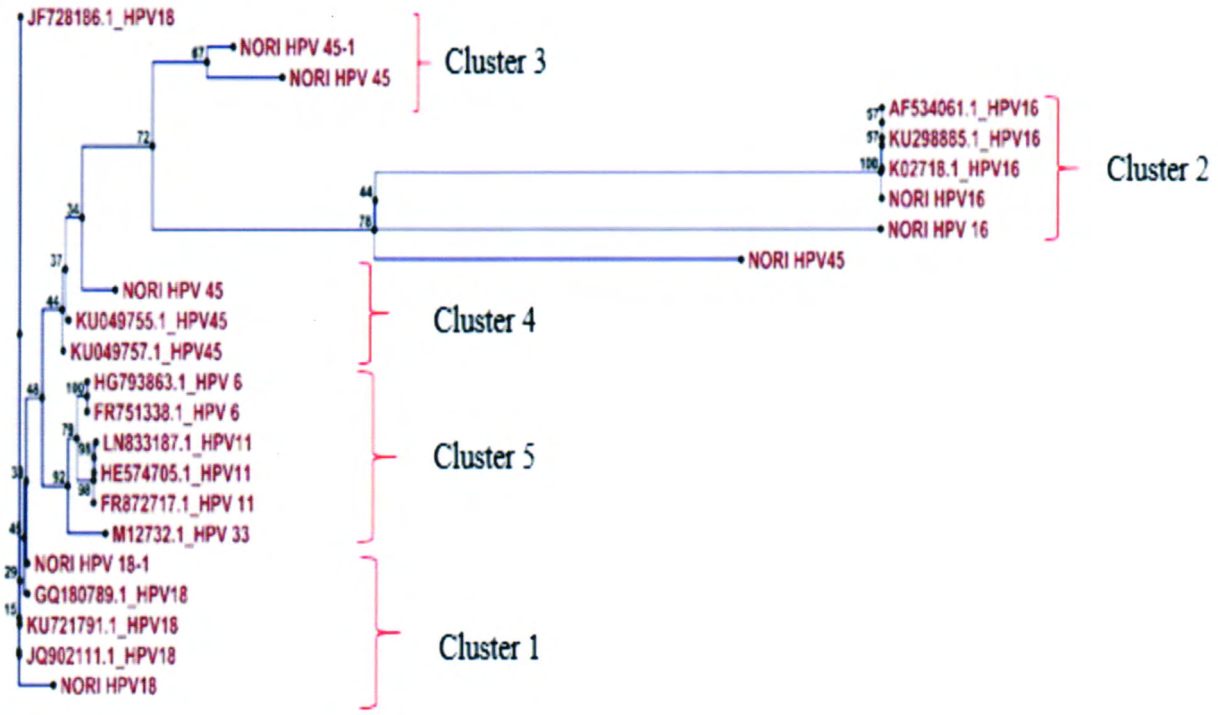


Figure 3.14 Phylogenetic Tree of HPV sequence by using Neighbour Joining method

Chapter 4

4. Discussion

Within the previous decade, the HIV/AIDS pandemic has overpowered the medicinal services frameworks and enormously affected females, especially those of conceptive age(Parham et al., 2006). The most vital danger component for obtaining of both HPV and HIV is sexual intercourse and so it is not astonishing that HPV contamination is extremely normal among HIV positive patients.HPV replication, severity, persistence and resistance to treatment of HPV related disease is enhanced as a result of HPV and HIV molecular interactions, along with reduced indigenous immune control of HPV infection with regards to HIV immunosuppression. HIV/AIDS patients not just have a higher pervasiveness of HPV. They all the more frequently have disease with various HPV types and also contamination with uncommon HPV subtype (Gormleyet al., 2009).

In the current study, the prevalence of HPV infection in HIV positive female patients visiting special HIV clinic at PIMS, Islamabad was assessed. According to the analyzed data, 30 (60%) HIV patients were positive for HPV infection. HPV type 16 was the most prevalent in HIV infected patients than other high risk types.The percentage of HPV 16 is 18% and HPV 18 is 10 % which is lower than the world wide prevalence of HPV 16 (31.9%) and HPV 18 (12.9%) (Clifford et al., 2006). The reason for this lower percentages is our society is more monogamously oriented than other societies.

In this study, the HPV was more prevalent in the age group of 21-30 years i.e. 50% of HIV patients. This increases the risk of persistent infection which may lead to the cervical cancer. The infection of HPV decreases with the increase of the age. Almost similar findings are reported in prevalence of human papillomavirus infection & cervicalabnormalities in HIV-positive women in eastern India by Chakravaty et al., 2016. In this study the premarital status of the patients were not studied because it was against the ethics of the doctor to practice the per speculum inspection until and unless they have severe issues. We can also say that the prevalence of HPV was higher in women of 20 to 35 age group are more sexually active as depicted by Manhart et al., 2006.

Ladies who have ever conceived a child have higher HPV predominance than ladies who have never conceived and expanding number of births have an expanded danger of HPV disease and

cervical carcinogenesis.Our results showed that females having >3 parity are more likely to be infected by the HPV.Our outcomes were in accordance with different studies (Castle et al., 2005; Zahid et al., 2005). According to the previous studies, the fact of increased HPV infection incident could be clarified by recurring injuries of cervix due to consecutive births. This can also be explained by the hormonal regulation during and after pregnancies causing the cervical changes(Hildesheim et al., 2001).The relationship between number of births and HPV pervasiveness has been affirmed by population studies where various births are normal (Munoz et al., 2002).

Ladies dwelling in the rural ranges were additionally at expanded danger of HPV disease in our study like a prior study from India by Chakravarty et al., 2016, 56.6% of the females that participated in our research were illiterate. Absence of education in females especially in village ranges can prompt the absence of information about their sexual wellbeing and keep them from affirming themselves in matter identified with sexual practices. This makes them powerless against hazardous sex prompting higher danger of procuring HPV disease.

In this study, we also observed the use and means of contraception. As these means are very helpful in the control of sexually transmitted diseases (Homes et al., 2004). The results illustrated that out of 30 positive patients, 33.3% patients used the barrier, 26.6% used oral, 13.3% used implinon and 10% used intra-uterine contraceptive devices as their mean of contraception. These results are in line with the previous study by Bosch et al., 2002.

According to the phylogenetic analysis, NORI HPV16 were grouped in the same cluster, which shows the high sequence similarity with other HPV variants in world. On the other hand, NORI HPV18 was clustered with sequences obtained from, Netherland (KU), China (JQ), Thailand (GQ) 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 closely linked to the two sequences NORI HPV 45 obtained in our study. But two of the NORI HPV Type 45 showed the variability in their sequences thus clustered in another group. The phylogenetic tree findings depicts the possibility of functional differences between variants within each type, which is important for epidemiological, etiological, pharmaceutical, and vaccination research.

Conclusionand Future Recommendations

We performed screening of HPV in HIV positive patients and found 60% prevalence of HPV in the population under study, thus we observed an association of HPV with HIV infection. Since HIV patients are immune-compromised and both HPV and HIV are transmitted through similar mode, we establish that HIV patients are at an increased risk of HPV infection, thus the prevalence was much higher as compared to studies conducted in non-HIV infected populations. Furthermore, we found high risk HPV 16 in 31.9% of the total HPV positive patients, while 12.9% contained high risk HPV 18. These results show that HIV positive patients are at a high risk of establishing cervical cancer. Thispopulation-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.

In Pakistan the screening of HPV is not practiced.People are still not well aware of the HPV and cervical cancer. Due to the lack of researches and data the risk of increase of HPV infection and cervical cancer is elevated. Even in developed countries, it is still a burden for scientists to diagnose and treat the cervical carcinoma. Since millions of women are infected, a novel prophylactic vaccination for HPV infection can help to decrease this burden globally. The proper awareness campaigns and screening methods should be designed for this purpose.

5. References

Alliance for cervical cancer prevention. (2009). New evidence on the impact of cervical cancer screening and treatment using HPV DNA tests,visual inspection, or cytology; Cervical cancer prevention fact sheet.

American Cancer Society. 2010, 1-9. Cervical cancer.

Anttila A, Katoniemi-Talonen L, Lainonen M, Hakama M, Laurila P, Tarkkanen J, Maila N, Nieminen P. (2010). Rate of cervical cancer, severe intraepithelial neoplasia and Adenocarcinoma in situ in primary HPV DNA screening with cytology triage; randomized study within organized program. *BMJ journal.* 340: 1804.

Bernard HU, Chan SY, Mànos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM.(1994). Identification and Assessment of Known and Novel Human Papillomaviruses by Polymerase Chain Reaction, Restriction-Fragment-Length Polymorphisms, Nucleotide Sequence, and Phylogenetic Algorithms. *J. Infect. Dis.*170:1077–1085.

BoschF X, LorinczA, MuñozN, MeijerC J L M, ShahK V. (2002). The causal relation between human papillomavirus and cervical cancer. *J ClinPathol.* 55:244-26.

C.M. D'Abramo and J. Archambault. (2011). Small Molecule Inhibitors of Human Papillomavirus Protein - Protein Interactions. *The Open Virology Journal.* 5:80-95.

Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, Daniel R, Shah KV, Sidransky D. (2000). Detection and Quantitation of Human Papillomavirus (HPV) DNA in the Sera of Patients with HPV-associated Head and Neck Squamous Cell Carcinoma. *Clinical Cancer Research.*6: 4171–4175.

Cappiello G, Garbuglia AR, Salvi R, Rezza G, Giuliani M, Pezzotti P, Suligoi B, Branca M, Migliore G, FormigoniPomponi D, D'Ubaldo C, Ippolito G, Giacomini G, Benedetto A.(1997). HIV Infection Increases the Risk Of Squamous Intra-EpithelialLesions In

Women With HPV Infection: An Analysis Of HPV Genotypes. *Int. J. Cancer.* 72:982–986.

Castle PE, Walker JL, Schiffman M, Wheeler CM.(2005). Hormonal contraceptive use, pregnancy and parity, and the risk of cervical intraepithelial neoplasia 3 among oncogenic HPV DNA-positive women withequivocal or mildly abnormal cytology. *Int J Cancer.* 117(6):1007-12.

Chan PK, Chan DP, To KF, Yu MY, Cheung JL, Cheng AF.(2001). Evaluation of extraction methods from paraffin wax embedded tissues for PCR amplification of human and viral DNA. *J ClinPathol.*54:401–403.

Chaturvedi A, Madeleine M, Biggar R, Engels E. (2009). Risk of human papillomavirus-associatedcancers among persons with AIDS. *J Natl Cancer Inst.* 101:1120 - 1130.

Cogliano VJ, Baan R, Straif K, Grosse Y, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-TallaaL, Guha N, Freeman C, Galichet L, Wild CP. (2011). Preventable exposures associated with human cancers. *JNCI.* 103: 1827-39.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zurHausen H. (2004). Classification of papillomaviruses. *Virology.* 324: 17–27.

Doorbar J, Foo C, Coleman N, Medcalf L, Hartley O, Prospero T, Napthine S, Sterling J, Winter G, Griffin H. (1997). Characterization of events during the late stages of HPV16 infection *in vivo* using high-affinity synthetic Fabs to E4. *Virol.* 238:40–52.

Ellerbrock TV, Chiasson MA, Bush TJ, Sun XW, Sawo D, Brudney K, Wright TC Jr. (2000). Incidence of cervical squamous intraepithelial lesions in HIV infected women. *JAMA.* 283:1031.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F.(2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 136: 359-386.

Gary M. Clifford, Maria Alice G, Gonc, Silvia Franceschi. (2006). Human

papillomavirus types among women infected with HIV: a meta-analysis. *AIDS.* 20:2337–2344.

GLOBOCAN 2012 (IARC), Section of Cancer Surveillance (23/4/2016)

Godfrey J. (2007). Towards optimal Health, Diane M. Harper, M.D., M.S., M.P.H. Discusses the HPV vaccine and prevention of cervical cancer. *Journal of women's Health.* 16(10):139-1401.

Groesbeck P. Parham, Vikrant V. Sahasrabuddhe, Mulindi H. Mwanahamuntu, Bryan E. Shepherd, Michael L. Hicks, Elizabeth M. Stringer, Sten H. Vermund. (2006). Prevalence and predictors of squamous intraepithelial lesions of the cervix in HIV-infected women in Lusaka, Zambia.*Gynecologic Oncology.* 103:1017–1022.

Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. (2007). Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet.* 370(9581):59-67.

Herbert J, Janis C. (2008). Reducing patient risk for human papillomavirus infection and cervical cancer. *Journal of the American osteopathic association.* 108 (2): 65-70.

Hildesheim A, Herrero R, Castle PE, Wacholder S, Bratti MC, Sherman ME, Lorincz AT, Burk RD, Morales J, Rodriguez AC, Helgesen K, Alfaro M, Hutchinson M, Balmaceda I, Greenberg M, Schiffman M. (2001). HPV co-factors related to the development of cervical cancer: results from a population-based study in Costa Rica. *Br J Cancer.* 84(9):1219-26.

Hing E, Saraiya M, Roland KB. (2011). Liquid-based Cytology Test use by office-based physicians.United States, 2006-2007. *National Health Statistics.* 40: 2-8.

Holmes KK, Levine R, Weaver M. (2004). Effectiveness of condoms in preventing sexual transmitted infections. *Bull World Health Organ.* 82(6):454-61.

Jaya Chakravarty, AnkitaChourasia, Minaxi Thakur, Abhishek Kumar Singh, Nisha Rani Agrawal, ShyamSundar. (2016). Prevalence of human papillomavirus infection &

cervical abnormalities in HIV-positive women in eastern India. *Indian J Med Res.* 143:79-86.

Jemal A, Bray F, Center M,Ferlay J, Ward E, Forman D. (2011). Global cancer statistics.CA. *A cancer journal for clinicians.* 61(2): 61-90.

Kerkar R, Kulkarni Y. (2006). Screening for cervical cancer: an overview. *Journal of obstetrics and Gynecology of India.* 56(2): 115-122.

Khurshid Anwar, Manabu Inuzuka, TaizoShiraishi, Kazuya Nakakuki. (1991). Detection of HPV DNA in Neoplastic and Non-Neoplastic Cervical Specimens from Pakistan and Japan by Non-Isotopic In Situ Hybridization.*Int J Cancer.* 47(5):675-80.

Koutsky L. (1997). Epidemiology of genital human papillomavirus infection. *Am J Med.* 102:3–8.

Kreimer AR, Clifford GM, Boyle P, Franceschi S. (2005). Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev.* 14:467–75. 7.

Lacey CJ, Lowndes CM, Shah KV. (2006). Chapter 4: burden and management of non-cancerous HPV-related conditions: HPV-6/11 disease. *Vaccine.* 24(3):35–41.

Lehtinen M, Ault K, Lyytikainen E, Dillner J, Garland S, Ferris D, Koutsky L, Sings H Lu S, Haupt R, Paavonen J. (2011). Chlamydia trachomatis infection and risk of cervical intraepithelial neoplasia. Sexually transmitted infectios. *BMJ Journal.* 87:372-376.

Levi JE, Fernandes S, Tateno AF, Motta E, Lima LP, Eluf-Neto J, Eluf-Neto J, Pannuti CS. (2004). Presence of multiple human Papillomavirus types in cervical samples from HIV-infected women. *GynecolOnco.* 92(1):225-31.

Likes W, Itano J. (2003). Human papillomaviruses and cervical cancer; not just a sexually transmitted diseases. *Clinical journal of oncology Nursing, USA.* 7 (3): 271-306

Manhart LE, Holmes KK, Koutsky LA, Wood TR, Kenney DL, Feng Q, Kiviat NB. (2006). Human papillomavirus infection among sexually active young women in the United States: implications for developing a vaccination strategy. *Sex Transm Dis.* 33:502-508.

McKenzie ND, Kobetz EN, Hnatyszyn J, Twiggs LB, Lucci III JA. (2010). Women with HIV are more commonly infected with non-16 and -18 high-risk HPV types. *GynecolOncol.* 3: 116(3):572-7.

Munoz N, Castellsague X, de Gonzalez AB, Gissmann L. (2006). Chapter 1: HPV in the etiology of human cancer. *Vaccine*. 24(3):S3/1–10. 4.

Muñoz N,Franceschi S,Bosetti C, Moreno V, Herrero R, Smith JS, Shah KV, Meijer CJ,Bosch FX; International Agency for Research on Cancer Case Study Group.(2002). Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. *Lancet*. 359(9312):1093-101.

Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group.(2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 348:518–27. 3.

Nouvo J, Melnikow J, Howell L. (2001). New tests for cervical cancer screening. *Journal of the American academy of family physicians*. 64: 5:780-786.

Nubia Munoz, Xavier Castellsague, Amy Berrington de Gonzalez, Lutz Gissmann. (2006). HPV in the etiology of human cancer. *Vaccine*. 24S3 S3/1–S3/10.

Outlook September 2000. Preventing cervical cancer in low resource setting.

Özgul N. (2009). The state of cervical cancer in turkey and cervical cancer screening studies; Ankara, Etlik Maternity and gynecology training and research Hospital, Ministry of Health, Turkey, pg 349.

P.D. Blumenthal, M. Lauterbach, J.W. Sellors, R. Sankaranarayanan.(2005). Training manual "cervical cancer prevention for low resource setting". JHPIEGO- an affiliate of Johns Hopkins University

Palefsky J. (2009). Human papillomavirus-related disease in people with HIV.*CurrOpin HIV AIDS*. 4(1):52–56.

Parkin DM, Bray F. (2006). Chapter 2: the burden of HPV-related cancers. *Vaccine*. 24(Suppl 3):S3/11–25. 5.

Parkin DM, Iscovich J.(1997). Risk of cancer in migrants and their descendants in Israel: II. Carcinomas and germ-cell tumors. *Int J Cancer*.70(6): 654-60.

Peng RR, Li HM, Chang H, Li JH, Wang AL, Chen XS. (2012). Prevalence and genotype distribution of cervical human papillomavirus infection among female sex workers in Asia: a systematic literature review and meta-analysis. *Sex Health* . 9(2):113-9.

Rachel H Gormley, Carrie L Kovarik. (2009). Dermatologic manifestations of HPV in HIV-infected individuals. *Current HIV/AIDS Reports*. 6:130–138.

SA Raza, S Franceschi, S Pallardy, FR Malik, BI Avan, A Zafar, SH Ali, S Pervez, S Serajuddaula, PJF Snijders, FJ van Kemenade, CJLM Meijer, S Shershah and GM Clifford. (2010). Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. *British Journal of Cancer*.102: 1657 – 1660.

Saeed Khan, Nadeem N. Jaffer, Mohammad Nasherwan Khan, Mohammad A. Rai, Majid Shafiq, Adnan Ali, Shahid Pervez, Nusrat Khan, Aliya Aziz, Syed H. Ali. (2007). Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma. *International Journal of Infectious Diseases*. 11: 313—317.

Susan Hariri, Elizabeth R. Unger, Maya Sternberg, Eileen F. Dunne, David Swan, Sonya Patel, and Lauri E. Markowitz. (2011). Prevalence of Genital Human Papillomavirus among Females in the United States, the National Health and Nutrition Examination Survey, 2003– 2006. *JID*.204.

SV Graham. (2010). Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies.*Future Microbiol.* 5(10): 1493–1506.

UNAIDS 2003. HIV/AIDS and Young People: Hope for tomorrow. Viewed 15.11.2007. http://data.unaids.org/publications/IRC-pub06/jc785.

UNAIDS 2006. Global Facts and Figures, viewed 18- November 2007. http://data.unaids.org/pub/GlobalReport/2006/200605 FS_globalfactsfigures_en.pdf.

WHO 2004. The Global Burden of Disease. Viewed 24 .02.2009. http://www.who.int/healthinfo/global_burden_disease/GBD_report_20 04update_full.pdf.

Woodman CB, Collins SI, Young LS. (2007). The natural history of cervical HPV infection: unresolved issues. *Nat Rev Cancer.*7: 11-22.

Zahid B, Khawaja N, Tayyeb R. (2005). Prevalence of abnormal cervical cytology and its relation with age and parity. *Ann King Edward Med Coll.* 11 (4): 524-5.