Association of Human Papillomavirus with Cervical Intraepithelial Neoplasia and Cancer

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Declaration

This thesis is titled "Association of Human Papillomavirus with Cervical Intraepithelial Neoplasia and Cancer". It is submitted in partial fulfillment of the requirement for the degree of Doctor of philosophy (Ph.D.) under the Faculty of Postgraduate Medical Science and Research, Dhaka University. To the best of our knowledge no part of it has been presented previously for another degree under this or any other university or institute.

This research work has been carried out in the Department of Obstetrics and Gynaecology, Virology and Histopathology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbag, Dhaka; Cancer Institute, Dhaka and Department of Immunology of BIRDEM, Shahbag, Dhaka, Bangladesh.

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Dedicated to the

ever alive memory of

(Late) **Mrs Meherunnessa,**

my Grandmother

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Date.....

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ABBREVIATIONS

ASCUS	Atypical squamus cells of undetermined significance
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Diseases
BMRC	Bangladesh Medical Research Council
BSCC	British Society of Clinical Cytology
BSMMU	Bangabandhu Sheikh Mujib Medical University
CA	Calibrator A
CB	Calibrator B
C.F.	Complement-fixing
CI	Confidence Interval
CIN	Cervical intraepithelial neoplasia
CIRH	Cancer Institute and Research Hospital
CIS	Carcinoma *in situ*
CPC	Combined probe cocktail
C. trachomatis	*Chlamydia trachomatis*
df	Degree of freedom
DNA	Deoxyribonucleic acid
DR 1	Detection reagent 1
DR 2	Detection reagent 2
DVI	Direct visual inspection
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
EV	Epidermis dysplasia verruciformis
FDA	Food and Drug Administration
FIGO	International Federation of Gynaecology and Obstetrics
FISH	Filter *in situ* hybridization
HC	Hybrid Capture
HC II	Hybrid Capture II
HGSIL	High grade squamous intraepithelial lesion
HPV	Human papillomavirus
HRHPV	High risk HPV
HSV II	Herpes simplex virus type II
IARC	International Agency for Research on Cancer
IBSCC	International Biological Study on Cervical Cancer
ICC	Invasive cervical cancer
IgG	Immunoglobulin G
IPGMR	Institute of Post Graduate Medicine and Research
ISH	*In situ* hybridization
LGSIL	Low grade squamous intraepithelial lesion
LLETZ	Large loop excision of transmembrane zone
LM	Light microscope
LRHPV	Low risk HPV
min	minutes
NC	Negative control

NCI	National Cancer Institute
N. gonorrhoea	*Neisseria gonorrhoea*
OD	Optical density
OPD	Out patient department
OR	Odds ratio
ORF	Open reading frame
Pap smear	Papanicolaou smear
PCR	Polymerase chain reaction
RLU	Relative light unit
RNA	Ribonucleic acid
RR	Relative risk
RRP	Recurrent respiratory papillomas
SBH	Southern blot hybridization
SD	Standard deviation
SPSS	Statistical package for the social sciences
STA	Sexually transmitted agent
STD	Sexually transmitted disease
STM	Specimen transport medium
T. pallidum	*Treponema pallidum*
TPHA	Treponema pallidum haemagglutination assay
TZ	Transformation zone
URR	Upstream regulatory region
USA	United States of America
VIA	Visual inspection of cervix after application of acetic acid
μ	micro (one thousand of a part, volume or time)

ABSTRACT

Introduction: Cervical cancer is the second most prevalent cancer among women worldwide after breast cancer, and the most common cancer among women in many developing countries. It constitutes about 20-30% of all cancers in female and it progresses from pre-invasive cervical intraepithelial neoplasia (CIN) to invasive cervical cancer (ICC). The aetiological factors of cervical cancer includes environmental, social, sexual and sexually transmitted agents (STAs) including Human papillomavirus (HPV).

Objectives: The objectives of this study were determination of the cervical prevalence of HPV in the married population, identification of the association of CIN and ICC with high and intermediate risk HPV (HRHPV) and low risk HPV (LRHPV) and to find the association of CIN and ICC with different sociodemographic parameters.

Methodology: This case-control hospital-based study included women with ICC (N=120) and CIN (N=70). ICC cases were diagnosed on the basis of histopathology. They were recruited from Bangabandhu Sheikh Mujib Medical University (BSMMU) and Cancer Institute and Research Hospital of Dhaka. The CIN cases were diagnosed on the basis of colposcopy directed biopsy and histopathology report and recruited from BSMMU. The age-matched control group (N=121) was randomly recruited from the women with normal cervix (examined by cervical smear and colposcope) attending BSMMU. All the subjects were non-pregnant between 22 to 70 years of age and were recruited during the period of October 1999 to May 2002. Each subject was interviewed about education, occupation, income, sexual behavior and reproductive history. Cervical samples and sera were collected from all three groups of women. All cervical samples were tested for HPV DNA using Hybrid Capture II (HC II) to detect HRHPV (type 16/18/31/33/35/39/45/51/52/56/58/59/68) and LRHPV (type 6/11/42/43/44). Presence of *Neisseria gonorrhoea* was checked by the detection of DNA of *N. gonorrhoea* from the cervical samples of CIN and control group by HC II. Presence of *Treponema pallidum* and Herpes simplex virus type II (HSV II) was detected by Treponema pallidum heamaglutination (TPHA) and identifying IgG antibidy (ELISA) respectively.

The data was analysed using Statistical Package for the Social Sciences (SPSS) program software. The relative risk (RR) as estimated by the odds ratio (OR) was the measure of independent association of HPV and different socio-demographic and reproductive factors in developing CIN and ICC was assessed by regression analysis.

Results: Among 120 ICC (Mean age, 47.18±11.24) cases, 94.2% were invasive squamous cell carcinomas, 5% were adenocarcinomas and 0.8% was adenosquamous cell carcinomas. Among 70 women with CIN (Mean age, 34.90±8.16), 34 (48.6%) had CIN I and 36 (51.40%) had CIN II or CIN III. Of the 120 ICC cases, 55.8% came from rural areas and 44.2% from urban areas. Out of 120 ICC cases, 25 (20%) were under the age of 40 and 95 (80%) were above that age. Incidence of ICC was very low (5%) under the age of 30.

HRHPV DNA was detected in 96.7 % of all ICC, 60.0% of different grades of CIN and 4.1% of control women. On the contrary, LRHPV DNA was found in 10.8% of all cervical carcinomas, 22.9% of different grades of CIN and 1.7% of control women.

Among these 36 cases of CIN II and III, 30 (83.3%) were HRHPV positive and this prevalence was significantly higher than that (35.3%) of CIN I (p=.000).

Considering all three groups of women together, the prevalence of HRHPV was significantly higher among the woman with lower level of education (p=0.000), women who were married in earlier years of their life (p=0.000), women who had 1st delivery between 12-15 years of age (p=0.007).

When all the STAs were included in regression analysis, it was observed that the influence of HRHPV for development of CIN (OR=27.1, 95% CI 9.6-77.0) and ICC (OR=893.7, 95% CI 211.1-3784.0) remained a strong and independent influencing factor. The influence of LRHPV for development of CIN (OR=6.5, 95% CI 1.1-38.2) still remained as an influencing factor but it did not show any independent influence on development of ICC (OR=1.8, 95% CI 0.3-11.2). Though *N. gonorrhoea* showed influence on development of CIN by bivariate analysis, it did not show any independent influence.

Most (68.3%) of the women of ICC group never attended school and only a few (5%) of them attended secondary or higher education. The lower educated group was 3.3 times (95% CI 1.1-10.1) more likely to develop ICC compared to the better educated group. The lower income

group (<Tk.3000/ per month) was 3.3 times (95% CI 1.2-9.2) more likely to develop CIN and 6.9 times (95% CI 2.1-23.2) more likely to develop ICC compared to higher income group. Majority of the women in all three groups were housewives and there was no association between occupation of the couples and cervical cancer.

The mean age (Mean±SD) at 1st marriage was 13.5±2.5, 15.6±3.5, and 15.7±3.5 years in the ICC, CIN and control group respectively and early marriage had significant association with cervical cancer ($p < 0.001$). The number of marriage of woman and their husband did not show significant association with cervical cancer. Early age of first marriage (OR=7.0, 95% CI 1.2-40.7) showed independent influence on development of ICC.

The mean age (Mean±SD) at 1st child birth was 16.5±2.9, 17.9±3.3 and 18.3±3.6 years in the ICC, CIN and control group respectively. Early age at 1st delivery (OR = 2.6, 95% CI 1.0-6.8) and parity eight and above (OR =17.7, 95% CI 1.4-228.1) were seen to have independent risk of developing ICC.

In the present study, oral pill was the most widely used contraceptive method among all three groups of women (48.06%), and none of the contraceptive methods showed independent influence in developing CIN and ICC.

All the results show very strong association of HRHPV with ICC. Contrarily LRHPV did not show any effect on development of ICC.

Conclusion: HRHPV infection was found to be the strongest determinant for development of cervical carcinoma and its precursors in Bangladesh. LRHPV has influence in developing CIN and no influence in developing ICC. Poor socioeconomic condition, low education level, early age of marriage and 1st delivery, multiparity (>8) were independently responsible for the development of ICC and independent influence of these factors were not effected by the strong influence of HRHPV. Development of awareness regarding natural history of cervical cancer, new guidelines for cervical cancer screening strategies can be developed from the information obtained from this study. A population based study should be carried out with larger sample size for further exploration of effects of STAs as syphilis and evaluation of cervical cancer screening strategies.

CHAPTER 1

INTRODUCTION

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1.1 Background

Cervical cancer is the second most prevalent cancer among women worldwide. An estimated 468,000 new cases of cervical cancer and 233,000 deaths occurred in the year 2000 and almost 80% of the cases of cervical cancer happen in the developing countries where, in many regions, it is the most common cancer among women (Parkin et al 2001, Parkin1992). The highest incidence rates are observed in Latin America and the Caribbean, sub-Saharan Africa and South and South East Asia (Parkin et al 2001). Among the South East Asian countries, Bangladesh and India have annual incidence of 11956 and 125952 respectively (Ferlay et al 2001). Global data showed that cervical cancer is the most common cancer among women in many developing countries and it constitutes about 21- 35% of the female cancer in different areas of India, the neibouring country of Bangladesh (Kulkarni et al 1996, Banerjee et al 1994, Sharma et al 1994).

Cervical cancer progresses slowly from pre-invasive cervical intraepithelial neoplasia (CIN) to invasive cervical cancer (ICC). In Bangladesh, so far the prevalence of cervical cancer and CIN has not been established from any population based study. However, data from the hospital statistics indicate that cervical cancer is a major health problem among the Bangladeshi women and constitutes about 22-29% of the genital tract cancer in different areas of the country (Akhter et al 1998, Akhter et al 1996, Kamaluddin et al 1993, Huq 1988, Rahim et al 1977). The diagnosed cases are managed mostly in few institutes and tertiary level hospitals either by surgery or radiotherapy or combined therapy. The high number of cervical cancer and inadequate treatment facilities demand the need of screening of this cancer and it's prevention in Bangladesh.

Cervical cancer is preceded by precancerous changes which represent a continuum of morphologic change beginning with CIN 1 (mild dysplasia) and progressing through CIN II (moderate dysplasia) and CIN III (severe dysplasia) to invasive carcinoma. They may not invariably progress to cancer and a good number of them may spontaneously

regress. The persistence and severity of the precancerous change influence the progress of the disease. The likelihood of regression of CIN I, CIN II and CIN III is 60%, 40%, 33% respectively and progression to ICC is 1%, 5% and greater than 12% respectively. The time lag between infection and development of ICC varies and is apparently on average more than 15 years (Ostor 1993).

Many of the studies tried to look into the aetiology of cervical cancer which included environmental, social, sexual factors and infectious agents. Wynder et al (1954) stressed on the importance of economic and sexual factors such as early marriage and sexual activity, multiple sexual partners and penile hygiene. A number of researchers suggested the possibility of sexual factors and sexually-transmitted etiology of cervical cancer (Hildesheim et al 1993, Martin 1967, Rotkin 1967, Christopherson et al 1965). Among the sexually transmitted agents, attention was paid to search the viral aetiology of cancer cervix. Naib et al (1969) first noticed the association of cervical carcinoma and active herpetic infection. Though viral aetiology of cervical cancer came into view in 1960s, main focus was paid on Herpes simplex virus type II (HSV II) and infection of genital tract by Human papillomavirus (HPV) was never suspected as a cause of cervical cancer before 1970s.

Zur Hausen (1977) for the first time suggested the association between HPV and genital cancer. But it received little attention from researchers and epidemiologists prior to 1988. Afterwards, several studies established HPV infection as a sexually transmitted disease (STD) and major risk factor for development of CIN and ICC (Liaw et al 1999, Lie et al 1997, Cuzick et al 1994, Schiffman et al 1993, Cox et al 1992, Koutsky et al 1992, Lorincz et al 1992, Howley 1991, Zur Hausen 1991, Reeves et al 1989). Studies revealed that cervical dysplasia and cancer usually arises among women with persistent HPV infection (Ho et al 1995, Hildesheim et al 1994). By the year 1992, HPV has been clearly established as the primary cause of cervical cancer in nearly all cases (Munoz et al 1992, Schiffman 1992). A study in 1999 estimated that over 99% of cervical cancers worldwide contained HPV DNA (Walboomers 1999).

Lorincz et al (1992) and cuzick et al (1994) categorized about 30 types of HPV that infect the genital mucosa into low, intermediate and high risk groups based on their

relative distribution in various histopathological diagnosis categories. "Low risk" HPVs (6/11,42,43 and 44) were present in 20.2% of low grade squamous intraepithelial lesions (LGSIL) but absent in cancers. "Intermediate risk" HPVs (31, 33,35,51,52, and 58) were detected in 23.8% of high-grade squamous intraepithelial lesions (HGSIL) and 10.5% of ICCs. "High risk" (HPV 16) were associated with 47.1% of both HGSILs and ICCs. "High risk" (HPV 18, 45, and 56) were found in 26.8% of ICCs. Numerically, HPV 16 was also the most important single type, detected in ICCs and HGSIL (Lorincz et al 1992). Therefore, it is revealed that 10-15 types of high and intermediate risk HPV (HRHPV) types were responsible for more than 90% of ICCs and they were referred to as cancer-associated HPVs (Bosch et al 1995, Lorincz et al 1992).

Cervical cancer gives opportunities for its control through screening because cervix is a surface organ, easily accessible and it has a long pre-malignant phase. If CIN are identified and successfully treated, the lesions do not develop into ICC. At present methods available for cervical cancer screening includes cytology-based screening, colposcopy, unaided visual inspection including visual inspection of cervix after application of acetic acid (VIA), aided visual inspection (e.g. gynoscopy) and tests for HPV markers.

Cytology-based screening using Papanicolaou smear (Pap smear) is the established method of cervical cancer screening, widely used and available screening instrument for detection of CIN. The test is designed to detect abnormal cervical cells. The procedure involves scraping cells from the cervix and then smearing and fixing them on a glass slide. The slides are then sent to a cytology laboratory and evaluated by a trained cytologist or cytotechnician to determine cell abnormalities. The concept of reduction of mortality and morbidity rate by cytology based screening programme is supported by results of this programmes (Queen et al 1999, Guzick 1978, Macgregor and Teper 1978). These studies have shown significant reductions in the incidence of ICC in screened populations. In general in many developed countries, women are advised to have their first Pap smear test soon after becoming sexually active and subsequently every one to three years. Compared with women in developed countries, very few women in developing countries have access to the screening programme for diagnosis of pre-cancerous lesions. _

During colposcopy a binocular magnifying instrument examines the surface features and vascular pattern of the cervical and vaginal epithelia. It magnifies the view of the surface of the cervix and, in most patients, of the endocervical canal, and allows precise delineation of the size and distribution of the neoplastic epithelium. Colposcopy directed biopsies can be taken from the most severely affected areas if required and sent for histopathological examination.

VIA has been tried to reduce the need of colposcopy. VIA involves swabbing the cervix with three to five percent freshly prepared acetic acid (vinegar) solution and examination of cervix in good light. Acetic acid application causes osmolar changes and water of the cells to leave and collapse of the cell membrane around the abnormal and enlarged nucleus. When exposed to this solution for few minutes, abnormal cells temporarily turn white and reveals aceto-white epithelium of the abnormal transformation zone (TZ). Many aspects of VIA make it hopeful approach for the use in low resource settings.

Tests for the detection of HPV can be used alone for cervical cancer screening or in a low resource setting as adjuvant with Pap smears or VIA to reduce the number of colposcopy. The epidemiologic study of HPV has been limited by HPV detection techniques. Even prior to 1980s HPV infection of cervix could be detected only by Pap smears or colposcopical examination of the cervix. A variety of nucleic acid hybridisation assays have been developed for detection of this infection from samples obtained from cervix. There are numerous HPV types and their carcinogenic potential vary among them and concentration has been focused for HPV tests to determine the HRHPV types in specimens.

Lorincz et al (1992) described two categories of HPV deoxyribonucleic acid (DNA) detection methods. One category uses amplification of nucleic acids and detects the products. This methods include polymerase chain reaction (PCR) based techniques. The other category identifies the nucleic acid directly and includes Southern blot hybridization (SBH), dot blot hybridization, *in situ* hybridization (ISH) and Hybrid Capture (HC) liquid hybridization (Digene Diagnostics).

There are two generation of HC test. The second generation detection method, Hybrid Capture II (HC II) is a linear signal amplified hybridization antibody capture microplate

assay. The HC II test involves a laboratory process that produces light signals roughly proportional to the amount of HPV DNA present in the specimen. Semiquantitative detection of eighteen types of HPV DNA in cervical specimens can be carried out and this test can differentiate between low-risk HPV (LRHPV) types (6/11/42/43/44) and HRHPV types (16/18/31/33/35/ 39/45 /51/52/ 58/59/68). HC II assay is standardized and highly reproducible and has been approved by United States Food and Drug Administration (FDA). For detection of high-grade dysplasia, the sensitivity range of this test has been recorded as 80-90% (Cuzick 2000, Manos 1999) and specificity has ranged from 57-89% (Cuzick 2000).

Though cervical cancer is a major health problem among the women of Bangladesh, it's screening programme has not been developed at the national level and screening is practiced sporadically by only few institutes, tertiary level hospitals and private practitioners. Hence lack of effective screening programme designed to detect and treat pre-cancerous conditions is the main reason for higher ICC incidence in Bangladesh. Therefore, this country needs to develop an efficient screening pragramme to reduce the incidence of cervical cancer and it's related mortality and morbidity. Prior to that, it is necessary to find out different risk factors related to this cancer in it's own environmental, social and cultural background. As HRHPV plays very important role in development of cervical cancer, worldwide attractiveness is widening to identify HRHPV infection as a part of cervical screening and also for treatment approach of CIN. There has been a number of alteration in the management schedule of CIN due to development of improved tests for identification of HPVs.

1.2 Rationale of the study

Treatment of cancer cervix is expensive and requires prolonged hospital stay, complicated operative procedures and radiotherapy. All the tertiary level hospitals and institutes of this country are carrying a huge load of cervical cancer patients. In Bangladesh, facilities for radiotherapy is available only in few institutions at the public sector and expensive at private sector. Cervical cancer is a disease of middle age group and therefore prevention and early diagnosis of this disease will significantly reduce the morbidity and mortality of the productive age group with potentiality to contribute to the society.

Cervical cancer is a slowly progressing tumour and it's natural history is reasonably well understood. It is now widely accepted that detection and treatment of pre-cancerous condition of the cervix reduces the incidence of frank cancer and there is significant reductions in the incidence of cervical cancer in screened populations. Therefore, implementation of cervical cancer screening programme has immense importance.

Though cytology based cervical cancer screening is an effective way of screening in developed countries, it does not test for the cause of the disease and only cellular abnormalities can be detected. Unfortunately this screening method is associated with a very high false-negative rate even up to 20%-40% (Fahey et al 1995, Gay et al 1985, Coppleson and Brown 1974). There may be more subjectivity and variability due to sampling errors (prone to false negative due to presence of blood, mucus and inflammation), difference of collection device (Martin-Hirsch et al 1999), sample preparation errors (fixing, staining etc). Koss (1989) also mentioned some important drawbacks of this screening programmes, such as too many screening rounds, over-reading of slides, and overtreatment of CIN. Apart from that implementation of cytology based screening programme needs an appropriate health system and set-up and the screening programme may be ineffective because of poor-quality cytology or inherent limitations due to a high rate of inflammatory and borderline smears. On the other hand, simplicity of performing HPV test makes the procedure operationally feasible. Sample for HPV can be collected by health worker and can be transported by post, courier service or other transport to the laboratory. The sample is stable at room temperature for couple of months. The use of calibrated, machine-read high technology screening systems

requiring limited number of trained personnel and comparatively more cost-effective than to establish as well as maintain cytology laboratories with highly trained cytotechnologists and physicians.

Routine colposcopic screening considered as extended gynaecological examination and can identify location and severity of the lesion. But it is expensive, time consuming and extensive expertise is necessary. It may create patient's anxiety and may lead to large number of unnecessary cervical biopsies and not suitable for routine use. Again detection of HPV DNA can play important role in reducing the number of colposcopy.

Studies showed that the sensitivity of atypical squamous cells of undetermined significance (ASCUS) can be improved by HPV DNA (Hybrid capture) detection (Solomon et al 2001, Wright et al 1998, Cox et al 1995, Hatch 1995). HPV DNA test is also useful in the diagnosis and management of cervical cancer in older women (Cuzick et al 1999), and for management of equivocal (borderline) cytology (Cox 1999, Wright et al 1998, Swygart 1997).

HPV DNA detection can help in the recognition and progress of CIN and therefore help for better management. Women with mild dyskaryosis are at high risk of developing ICC despite cytological follow up (Soutter & Fletcher 1994) and therefore these patients can be followed up by HPV DNA detection. Soutter et al (1977) mentioned the necessity of careful, long-term follow-up after conservative therapy of CIN. After conization and large loop excision of transformation zone (LLETZ) – there may be complete or incomplete remission. Studies proved that HPV DNA detection has important role for confirmation of completion of treatment and follow up of these cases (Lin et al 2001, Kjellberg et al 2000). The cases of discordance between cytology and colposcopy and the discordance between colposcopy and histopathology report can also be solved by HPV DNA detection.

Cervical cancer prevention programmes should be based on the knowledge of progression of the disease from precursor lesions to invasive state. Studies suggested that expectant management of CIN I was safe, cost-effective and without compromising patient care as spontaneous resolution of CIN I occurs in majority of the cases (Moscicki et al 2001, Falls 1999, Ho et al 1998). Therefore if CIN I are HRHPV DNA negative,

conservative management is justified. CIN II / III have the potential to progress to ICC and necessitate treatment. Therefore, HPV DNA test can be used for conservative management and follow-up of these CIN patients. Identification of HRHPV negative cases among dyskariotic, borderline or inadequate cervical smear can also help in minimizing unnecessary colposcopic examination. Studies mentioned about the use of HPV tests as a secondary triage in patients with smears suspecting mild or moderate dysplasia to reduce the number of unnecessary colposcopy (Nobbenhuis et al 1999, Walboomers et al 1999, Bollen et al 1997). Walboomers et al (1999) also mentioned about HPV DNA test as primary screening-either alone or in combination with cytology.

Cytology based screening needs a huge amount of colposcopy service backup. Colposcopy service is expensive, need travelling of patients to hospitals and it is available only in few tertiary centres of this country. Health care service in low resource settings like Bangladesh as well as families with low socioeconomic condition can not afford this extra expenditure. Bangladesh needs to develop a less expensive way of cervical cancer screening programme. VIA can be used in low resource setting and HPV test can be used as an adjuvant in suspected (VIA positive) cases to reduce the number of colposcopy. VIA is relatively simple, need minimum infrastructure support and part of the result of the procedure is available immediately. The high sensitivity of VIA shows that the test could be valuable in detection of CIN, but it's cost effectiveness is reduced by high numbers of false positive results (Graffikin et al 1999). So attention should be given to improve the specificity of VIA by identifying HRHPV in VIA positive cases.

Though HRHPV infection plays substantial role in developing cervical cancer and HRHPV DNA detection may play crucial role in various ways, so far learning is inadequate on the prevalence of HPV among Bangladeshi women with cervical cancer, CIN and normal cervix. It was necessary to identify the incidence of HPV infection of genital tract and its risk group in the female population of this country prior to a rational approach in developing cervical cancer screening programme and an appropriate line of management for CIN patients. Other factors which have been identified as responsible for development of cervical cancer such as early marriage and first child birth in younger age is also common in Bangladesh. Hence the influence of age of marriage, first child

birth, parity and socio-economic condition on the development of cervical cancer needed to be evaluated in this population.

In the developed countries, initially the acquired knowledge from different researches on HPV could not be applied to public health or patient care concerns, but later on improved laboratory techniques has been developed to make the knowledge useful for women's reproductive healthcare and cancer prevention. In Bangladesh, data on prevalence of HPV in ICC, CIN and normal cervix and introduction of HPV test may provide useful information to clinicians and policy makers in understanding the problem and contribute to public health. The obtained information will also be useful for the purposes of counseling, selection of therapy and follow up.

Bangladesh has a comprehensive health infrastructure which offers the possibility of introducing screening programmes. Furthermore, the highly successful family planning programme has encouraged awareness regarding women's health related issues and this could be beneficial in introduction of this new, potentially sensitive procedure.

As persistence of certain risk groups of HRHPV influence the progress of the disease, identification of the presence of this virus plays important role in screening, prevention and management of CIN. Understanding the prevalence of cervical cancer and it's related morbidity and mortality and the role of identification of HRHPV in the prevention of this disease, it seems to be very important to gather knowledge about HPV prevalence among the Bangladeshi women with cervical cancer, pre-cancer and normal cervix. Identification of other risk factors related to HPV and cervical cancer in the female population of this country with it's own social background is also important. In this study HC II was used for detection of HPV DNA of the cervical samples from 120 women with ICC, 70 women with CIN and 121 control women.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Literature review is crucial part of a study. An extensive literature search was performed. The available literature can be grouped as follows:

2.1 Precancerous conditions of the cervix (cytologic and histological issue)

Traditionally, precancerous changes represent a continuum of morphologic change and form a spectrum of disease beginning with CIN I (mild dysplasia) and progressing through CIN II (moderate dysplasia) and CIN III (severe dysplasia) to invasive carcinoma. They may not invariably progress to cancer and may spontaneously regress. The persistence and severity of the precancerous change influences the progress of the disease.

2.1. a. Classification of the cytological abnormalities related to CIN

The TZ between the columnar cells of the endocervix and the squamous epithelium of the ectocervix has been recognised as the most vulnerable site for development of CIN and ICC. Pap smear is collected from this area and abnormalities are detected by cytological examination. Over the last 50 years, the terminology used to classify abnormalities of the cervical smear has changed from time to time.

Two classification systems are used for cytological identification of cervical cancer precursor conditions. In the CIN system, cytologic findings consistent with mild cervical dysplasia is categorized as CIN I, moderate dysplasia as CIN II, and severe dysplasia including carcinoma *in situ* (CIS) as CIN III (Richart 1980). A Working Party of the British Society of Clinical Cytology (BSCC) in 1978 recommended the use of 'dyskariosis' and 'dyskariotic' to describe the nuclear abnormalities for cells suspected

from both CIS or even early invasive cancer (Spriggs et al 1978). Another report of the Working Party of the BSCC in 1986 classified dyskariotic cells as mild, moderate or severe depending on nuclear and cytoplasmic characteristics. Mild dyskariosis correlates with cells from the surface of CIN I. Severe dyskariotic cells correlate with cells from the surface of cervical epithelium showing CIN III or invasive carcinoma (Evans et al 1986).

National Cancer Institute (NCI) held a workshop in 1988 in Bethesda, Maryland and they developed another classification system for cytologic reporting known as the Bethesda System (Lundberg 1989). This system introduced the cytologic term atypical squamous cells of undetermined significance (ASCUS). In the Bethesda System (Table 3.1), potentially premalignant squamous lesions fall into three categories: low-grade squamous intraepithelial lesions (LGSIL) include CIN 1 (mild dysplasia) and cellular changes indicative of HPV infection, termed koilocytotic atypia. High grade squamous intraepithelial lesion (HGSIL) includes cytologic changes consistent with CIN II and CIN III (moderate dysplasia, severe dysplasia and CIS).

Table 2.1: Terminology Used for Cervical Abnormalities (Path 2001)

Bethesda System	**Cervical Intraepithelial Neoplasia (CIN) system**	**Common Dysplasia Terminology**
Atypical squamous cells of undetermined significance (ASCUS)	Cellular atypia	Unspecific cellular changes
Low-grade squamous intraepithelial lesions (LSIL)	CIN I	Mild dysplasia
High-grade squamous intraepithelial lesions (HSIL)	CIN II CIN III (including CIS)	Moderate and Severe dysplasia/CIS

Abnormal cells that do not fulfill the criteria for either LGSIL or HGSIL are described as ASCUS. This category includes many of the minor abnormalities that in the past were termed atypical. The ASCUS category does not include benign, reactive or reparative changes and these are coded as normal in the Bethesda System. Similarly, the ASCUS category does not include condylomatous or koilocytotic atypia, which are categorized as LGSIL.

2.1.b. Classification of the histopathological abnormalities of CIN

Colposcopical examination and colposcopically collected biopsy specimen from the cervix play important role to identify the actual pathological condition from a group of different categories of cervical cytological abnormality. The Colposcope magnifies the view of the surface of the cervix and, in most patients, of the endocervical canal, and allows precise delineation of the size and distribution of the neoplastic epithelium. Colposcopy directed biopsies are taken for histopathological examination from severely effected areas.

Histological diagnosis depends on cellular immaturity, cellular disorganization, nuclear abnormalities, and increased mitotic activity. The extent of these cytologic and histologic disturbances identify the degree of neoplasia.

Low grade SIL (CIN I) - If mitoses and immature cells are present only in the lower one-third of the epithelium, the lesion is designated as CIN 1. In this situation the cells throughout the full thickness of the epithelium show nuclear abnormalities and the cells in the upper and middle thirds of the epithelium undergo cytoplasmic differentiation. The cells in the lower third, or less are undifferentiated with loss of polarity.

High grade SIL (CIN II, CIN III) - Dysplasia involving the middle third, upper third or full thickness is diagnosed as CIN II, CIN III or CIS respectively. The cells in the upper third of the epithelium undergo a variable degree of stratification and of cytoplasmic differentiation.

2.2 Natural history of cervical cancer and role of HPV in carcinogenesis

A clear knowledge of the natural history of cervical cancer is essential to develop an effective screening method and follow-up strategies. It is also essential to know the current understanding of the natural history of cervical cancer in deciding when to initiate screening, how often to screen and when to recommend treatment and/or follow-up for evaluation.

In 1960s gynaecologists, pathologists and other researchers exerted considerable effort to search the aetiology of cervical cancer with help of colposcope (Richart 1965) and examinining colposcopy directed biopsy materials. Much attention was made on metaplastic process of covering epithelium of the cervix and its preneoplastic changes. The concept of coital factors (sexual variables) including married state, early age of first coitus, more marital and sexual mates, male sexual hygiene, circumcision status of coital partners and spermatozoa were suspected to have association with cervical cancer. The observed relationships of cervical cancer to these factors led a number of researchers to suggest the possibility of a sexually-transmitted etiology in this condition (Martin 1967, Rotkin 1967, Christopherson et al 1965).

Among the sexually transmitted agents (STAs), attention was paid to search the viral aetiology of cancer cervix. Investigations for identification of virus was poorly developed in 1960s and serological investigations for complement fixing antibodies against 11 viral antigens failed to explore association of virus with cervical cancer (Lewis et al 1965). Still different studies continued and Christian et al (1965) were able to isolate HSV from the malignant lesions. Josey et al demonstrated that HSV infection of the vulva, vagina and cervix was common among women of low socioeconomic condition. It has been suggested that the venereally transmitted HSV II may play an etiologic role in carcinoma of the cervix (Josey et al 1968, Josey et al 1966). This tentative assumptions remained hypothetical until Rawls et al (1968) isolated genital HSV II from four smegma specimens of young male venereal disease clinic patients. But the role of viruses in the etiology of cervical carcinoma was still an unexplored area prior to Naib et al (1969) noticed the association of cervical carcinoma and active herpetic infection. They

observed squamous atypia in 23.7% of biopsied cervices with cytologic evidence of active herpetic infection.

Though viral aetiology of cervical cancer came into view, main focus was paid on HSV II and infection by HPV was never suspected as a cause of cervical cancer before 1970s. Zur Hausen for the first time suggested the association between HPV and cervical cancer (Zur Hausen 1977). Figure 2.1 showed a hypothetical schema describing the multistep pathogenesis of cervical cancer.

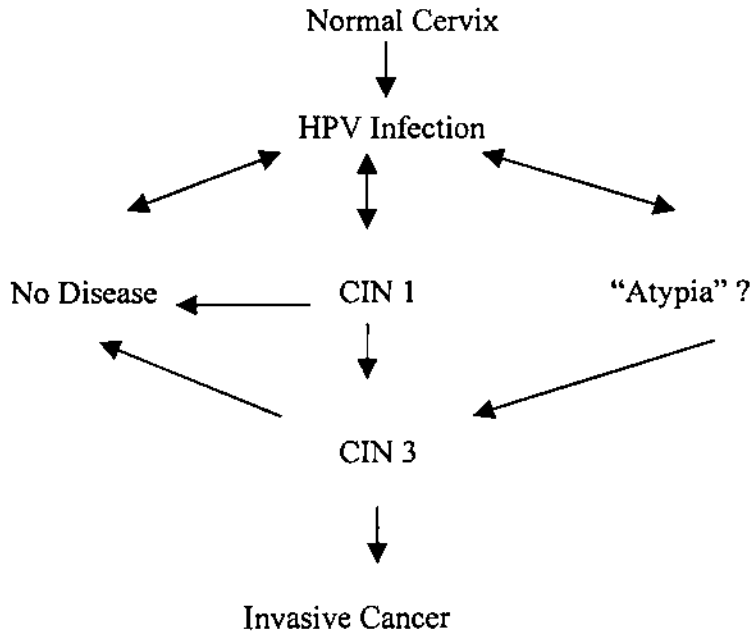


Fig 2.1: Hypothetical Schema Describing The Multistep Pathogenesis Of Cervical Cancer (Shiffman and Brinton 1995).

It has been universally recognized that papillomavirus causes infectious epithelial lesions in birds, animals and humans. Infection of women's genital tract by HPV generally occur in their teens, twenties, or thirties. The commonest presentation of infection is a self-limiting benign tumour named papilloma or wart. Most HPV infection of cervix, including those with cytologic abnormalities, resolve spontaneously within few months, returning to HPV DNA negativity (Hildesheim et al 1994). In some cases, persistence of infection occurs (Ho et al 1995, Hildesheim et al 1994) and cytological changes take

place which progress to CIN or ICC. HPVs are found in all grades of CIN, but have also been detected in normal cells. Therefore it is obvious that an atypical epithelium does not always progress to ICC and the possibility of invasion increases with the severity of the atypia. Even the higher degrees of atypia may regress in a notable proportion of cases.

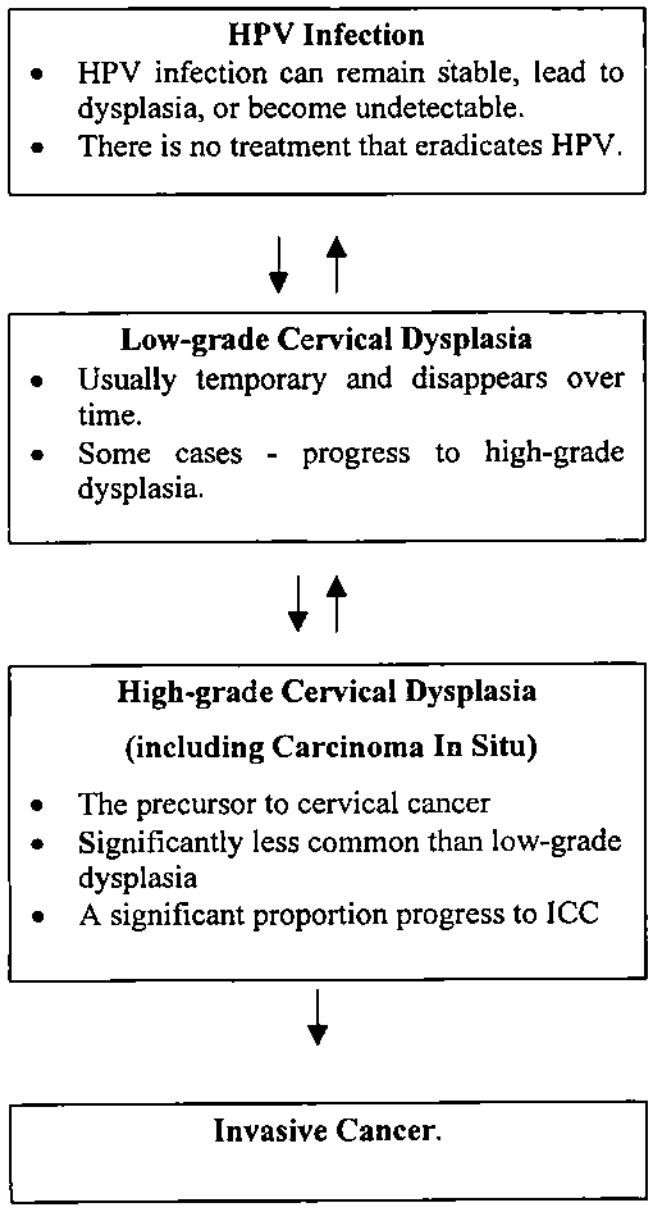


Fig 2.2: Genital HPV Infection and Development of Cervical Cancer (Modified from Out Look 2000)

Figure 2.2 showed the natural history of development of cervical cancer. Progression through different grades of CIN to CIS or ICC may take several decades. The time lag between infection and development of ICC is probably on average more than 15 years (Ostor 1993).

DE Villiers (1989) extensively reviewed the heterogenicity of HPVs. He mentioned about isolation and characterization of 60 different types of HPVs with the help of cloning systems in bacteria. Several other studies showed that only about 10-15 of the more than 30 genital tract HPV types were responsible for more than 90% of ICC (Bosch et al 1995, Lorincz et al 1992).

2.3 Human Papillomavirus

Research related to HPV did not get much attention until 1980s when by the development of *in vitro* transformation assays permitted the analysis of the viral functions involved in the induction of cellular proliferation.

Papillomavirus is a member of *Papovaviridae* family (Fig 2.3). The term papovavirus originated from the first two letters of three groups of viruses (rabbit papillomavirus, mouse polyomavirus, and simian vacuolating virus). The properties shared by these viruses include small size, a nonenveloped virion, an icosahedral capsid, a double-stranded circular DNA genome and the nucleus as a site of multiplication (Murphy and Kingsbury 1991). Members of the papillomavirus and polyomavirus subfamilies can be distinguished by differences in the size of the virions (55 nm versus 40 nm) and by the size (8,000 base pairs versus 5,000 base pairs) of the viral genomes (Howley 1991).

The papillomaviruses are widespread in nature and have been recognized primarily in higher vertebrates (Howley 1991, DE Villiers 1989). Viruses have been found from humans and animals. The papillomaviruses are highly species specific and induce squamous epithelial tumors and fibroepithelial tumors in their natural hosts. Most animal papillomaviruses are associated with purely epithelial proliferative lesions and have a specific cellular tropism for squamous epithelial cells. Histologically, the lesions induced by papillomaviruses share a number of features. In general, there is thickening of the

epidermis (acanthosis) and hyperkeratosis. Usually, there is some degree of papillomatosis. Keratohyaline granules are often prominent in the granular layer of the epithelium.

Papillomavirus of animal includes Shope papillomavirus (of rabbit), bovine papillomaviruses, and papillomaviruses of many animal species as horses, dogs, sheep, elk, deer, the harvest mouse and the multimammate mouse. Papillomavirus of human includes HPV 6 and 11 (associated with benign genital condylomas) and HPV 16,18, 31, 33, 35, and 39 (have a high correlation with genital and oral/pharyngeal carcinomas). HPV usually do not pass through the basement membrane of the epithelium and infect the underlying tissues indicates that interaction between virus and immune system happens mainly within the epithelium (Beckmann et al 1985).

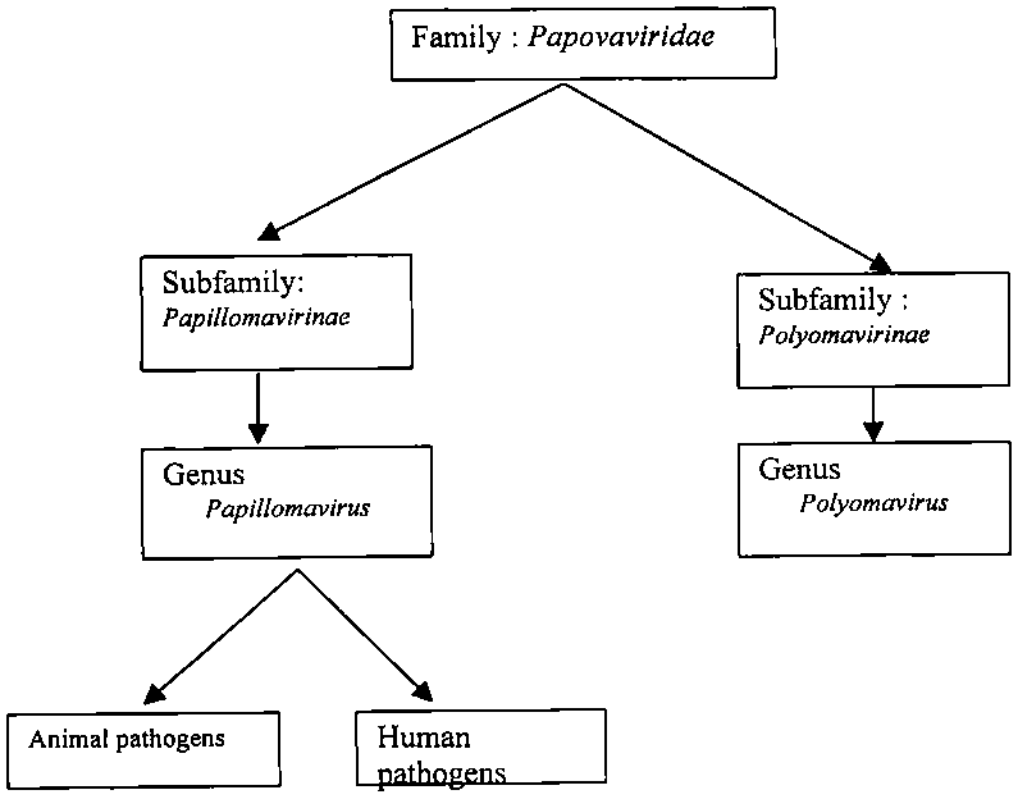


Fig 2.3: Family tree of Papovaviridae (Modified from Murphy and Kingsbury 1991)

2.3.a. Structure of HPV

Papillomaviruses are epitheliotropic DNA tumor viruses characterized by a double-stranded, circular DNA genome of 7800 to 7900 base pairs. These are small (45-52nm), non-enveloped viruses with icosahedral symmetry. The capsid comprises 72 capsomeres, and has species-specific antigenic determinants on the outer surface and genus specific determinants internally. The viral genome is organized into three major regions: (Park et al 1995) two protein-encoding regions (the early and the late gene regions) and a noncoding upstream regulatory region (URR).

Papillomaviruses share a genus-specific antigen (capsid antigen). In addition to the capsid proteins, this DNA codes for a small number of proteins necessary for its maintenance and replication. The genome organization of all the papillomavirus is similar, as is the function of the viral proteins (Fig 2.4 and Table 2.2).

The typical lifecycle of the papillomaviruses involves infection of the keratinizing epithelium. The viral genome replicates as an episome. In the replicating basal cells of epithelium, a maintenance replication of the viral DNA occurs with very little viral transcription. Differentiation of epithelial cells is required for increased replication and transcription and virion production. In HPV-induced precancerous and cancerous lesions, only viral proteins E6 and E7 (Table 2.2) are expressed, and no virus is made (Auborn 1998).

Table 2.2: Papillomavirus Proteins (Auborn 1998)

Viral Protein	Functions
E1	DNA replication
E2	Transcription regulation, DNA replication
E4	Alteration of cell skeleton
E5	Transformation
E6	Binding to p53, transformation
E7	Binding to pRb, transformation
L1	Major capsid protein
L2	Minor capsid protein

Early region

The early region (El-E8) encodes genes for virus replication and for host-cell transformation. The early region shows eight open-reading frames (ORFs). ORFs are DNA segments that are transcriptional units and are capable of encoding for protein. Of the eight ORFs, only E3, E4, and E8 have not been implicated in cell transformation. E1 and E2 ORFs are involved indirectly in transformation via their primary roles in replication and gene expression, respectively. ORFs E5, E6, and E7 contribute directly to cell transformation. Expression of early viral genes also gives rise to concomitant proliferation of the cells to form the benign lesion. Two of the early region ORFs, E6 and E7, encode for oncoproteins critical for viral replication as well as host cell immortalization and transformation. The transforming genes for HPV 16 and HPV 18 have been mapped to E6 and E7 ORFs. This is the region of the genome that is expressed in cervical carcinomas and in cell lines derived from ICCs that harbor integrated HPV 16 and HPV 18 genomes (Baker et al 1987, Schwarz et al 1985).

Late region

The late gene region contains two separate ORFs termed L1 and L2, which encode for the viral capsid proteins. Activation of late viral gene expression with synthesis of the capsid proteins and assembly of virus particles occurs in the outer layer of the epithelium, linked in some manner to the normal pattern of keratinization and terminal differentiation of the cells (Benjamin and Vogt 1991). Synthesis of capsid proteins, vegetative viral DNA synthesis and assembly of virions occur only in the terminally differentiated cells.

Upstream regulatory region (URR)

The URR does not encode for proteins, but instead, contains a complex array of overlapping binding sites for many different transcriptional repressors and transcriptional activators (Park et al 1995). Because of its potential for binding a wide array of specific transcriptional factors, the URR may play a critical role in determining the host range of specific types of HPV.

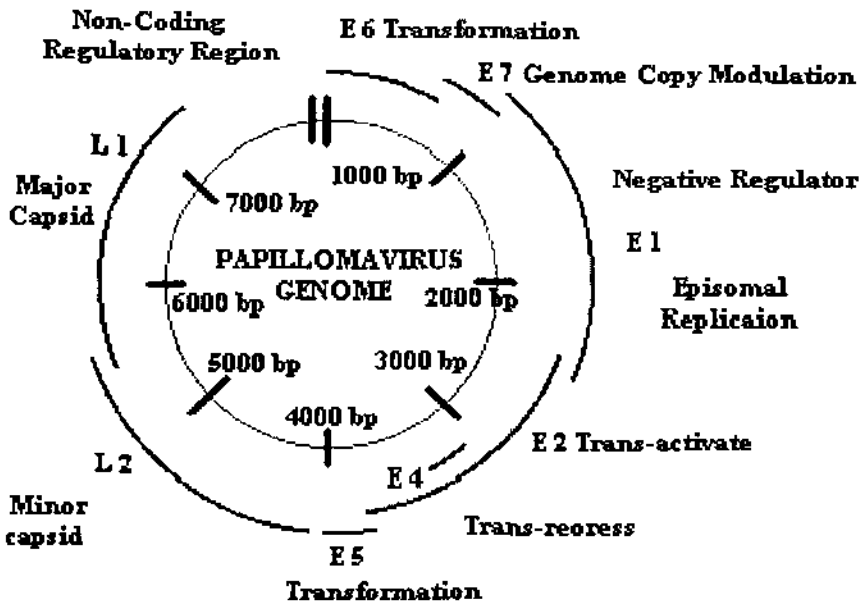


Fig. 2.4: The Papillomavirus Circular DNA Genome (Modifid from Auborn 1998, Koutsky et al 1988)

2.3.b. Classification of HPV

Biologically, the HPV types are divided into two groups: cutaneous and mucosal. Most lesions in animals are cutaneous, even though infections of mucosal squamous epithelium are not rare.

In spite of the fact that human and animal papillomaviruses share a similar genomic organization, they are extremely species specific and do not cross-infect other species.

Many types and subtypes of papillomaviruses may exist that infect a given species. However, classification of types is based on host range and relatedness of the viral genomes. A papillomavirus must have greater than 50% DNA homology to be considered the same type. Whenever less than 50% homology with the other known types of a papillomavirus for that species is recognised during hybridization under stringent conditions in liquid phase, a new type papillomavirus for that species is considered (Coggin 1979).

Cutaneous group

Cutaneous warts can often be distinguished morphologically. They are usually harmless and regress spontaneously within two years. They are transmitted by direct contact or from fomites. Large numbers of cutaneous warts may develop in immuno-deficient patients. Patients with epidermodysplasia verruciformis (EV) have impaired cell medicated immunity and develop multiple lesions carrying many HPV types not found in the general population (DE Villiers 1989).

Mucosal group

Mucosal group includes the HPV types that infect the respiratory, gastrointestinal and genital tract (DE Villiers 1989). Recurrent respiratory papillomas are caused by HPV 6 and 11. They are transmitted to infants during vaginal delivery, or in adult life through oral sex. children under five years are at highest risk of developing the disease, though onset may be delayed until later in life. They may progress to cancer, HPV 6 and 11 are also associated with squamous and inverted papillomas of the nasal cavities. Some of the HPV types associated with the genital tract are shown in Figure 3.5.

2.3.c. HPVs related to genital tract lesions

HPVs are classified according to their degree of genetic homology. The types are numbered sequentially when they are characterized. Each type has its own tissue predilection and disease spectrum. DE Villers (1989) mentioned that among the 60 individual HPV types isolated by that time more than 20 have been detected in pre-malignant and malignant genital lesions.

About 30 types of HPV that infect the genital mucosa have been categorized into low, intermediate and high risk groups depending on their relative distribution in various histopathological diagnosis categories (Cuzick et al 1994, Lorincz et al 1992).

Risk groups

HRHPV are found frequently in ICC and CIN. Lorincz et al (1992) revealed the presence of an oncogenic HPV conferred relative risks ranging at 65.1-235.7 for the occurrence of a high-grade lesion and 31.1-296.1 for an ICC. He also defined different categories of genital HPVs as high risk, intermediate risk, low risk HPVs.

High risk HPVs (HPV type 16, HPV type 18)

Historically, HPV 16 and HPV 18 have been regarded as high risk cancer associated HPVs. The complete nucleotide sequence of HPV 16 DNA was cloned from an ICC by Seedorf et al (1985). Fuchs et al (1988) examined colposcopy directed cervical punch biopsies from 362 women and HPV 16 was more abundant than other HPV types in HSIL and ICC (50%-60%), compared to healthy epithelium. A large multicenter randomised case-control study in Latin America detected HPV 16, 18 or both in 62% of the cases and 32% of the controls (Reeves et al 1989). Other studies also showed that HPV 16 is the most prevalent type among HSIL and ICC (Bosch et al 1995, Cuzick et al 1995, Cuzick et al 1994, Lorincz et al 1992).

Intermediate risk HPV types - 31/33/35/39/45/51/52/56/58/59/68.

HPV 31: Lorincz et al (1986) detected HPV 31 and it was most closely related to HPV 16. Although absent from all genital condylomas studied, HPV 31 was present in approximately 20% of mild and moderate dysplasias and 0.6% of ICCs .

HPV 33: Beaudenon et al (1986) reported the molecular cloning and characterization of HPV 33 from 4-8% of biopsies of CIN and ICC.

HPV 35: Lorincz et al (1987) identified HPV 35 DNA from an endocervical adenocarcinoma and it was prevalent in 1% CIN and 4% anogenital cancers.

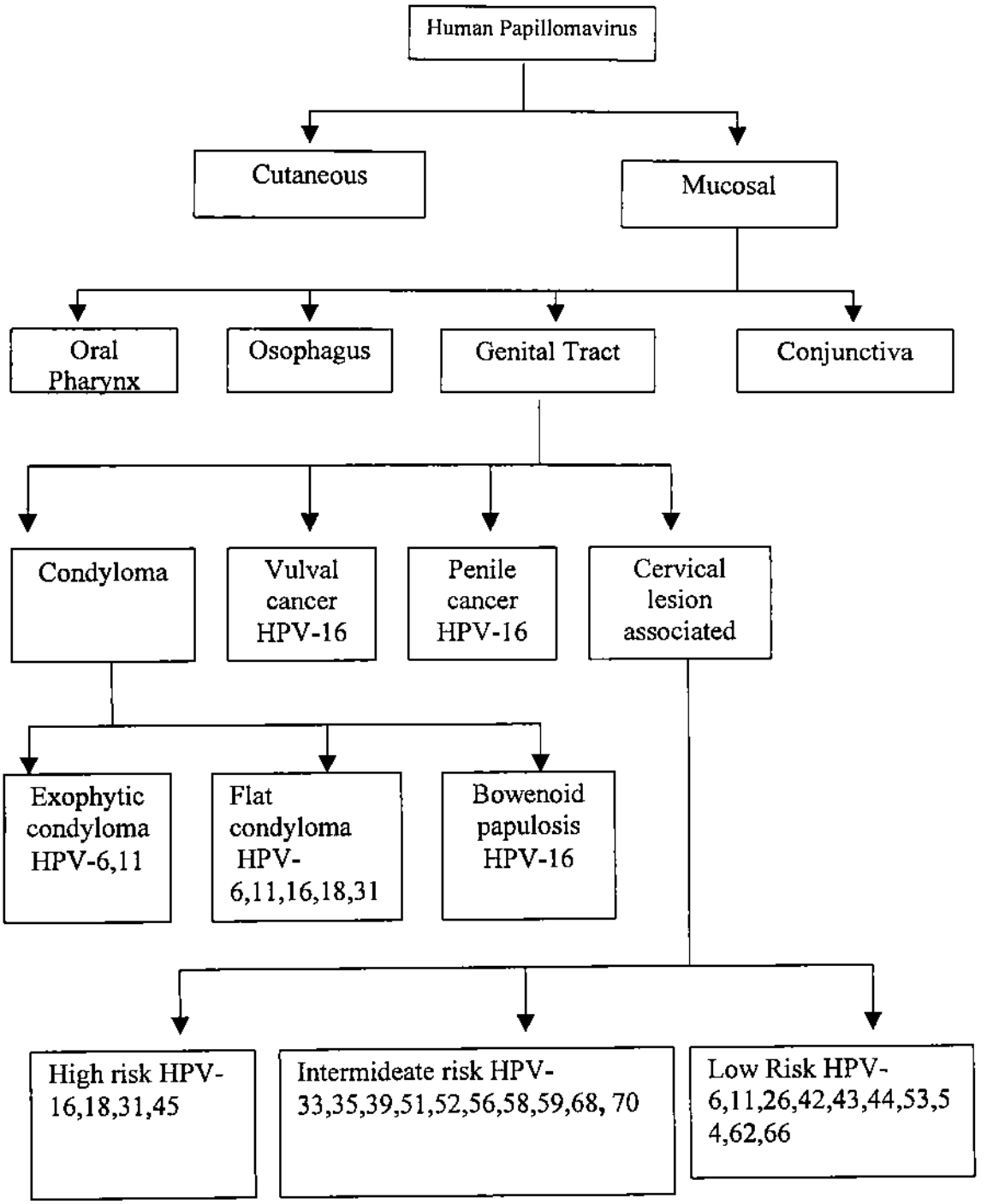


Fig 2.5: Classification of Human Papillomavirus (Modified from Lorincz et al 1992, Howley 1991, DE Villiers 1989)

HPV 39, HPV 42: The genomes of two new genital HPV types named HPVs 39 and 42, have been cloned from biopsy specimens of anogenital areas and among these two viruses, HPV 39 represented a potentially oncogenic genital HPV type (Beaudenon et al 1987).

HPV 45: DNA of HPV type 45, a new type 18 related HPV was cloned from a recurrent cervical lesion displaying mild to moderate dysplasia with koilocytosis (Naghashfar et al 1987).

HPV 52: Shimoda et al (1988) cloned HPV 52 in 2% of CIN and ICC.

HPV 56: HPV 56 was identified in two of 464 normal cervical tissues, in five of 227 cervical condylomas and CIN, and in two of 84 ICCs (Lorincz et al 1989).

HPV 68 and HPV 70: HPV 68 and HPV 70, were cloned from CIN I and vulvar papilloma, respectively. Both types were related to HPV 39, a potentially oncogenic virus (Longuet et al 1996).

Low risk HPV types 6/11/42/43/44

About a dozen of other genital HPV types may produce mild cytologic abnormalities, but are rarely found in HSIL or ICC (Borch et al 1995, Lorincz et al 1992).

HPV types 6/11: Dartmann et al (1986) isolated complete nucleotide sequence of HPV 11 DNA from genital warts (condylomata acuminata). Fuchs et al (1988) examined colposcopically directed cervical punch biopsies from 362 patients by SBH and HPV 6/11,16,18 and 31 were found in 2.9-13.7% of histologically normal epithelia and HPV 6/11 prevailed in CIN I.

HPV type 42: The genomes of HPV 42 have been cloned from biopsy specimens of anogenital areas along with HPV 39. HPV42 belongs to the low-risk group of genital HPVs (Beaudenon et al 1987).

HPV 43, HPV 44: Cloning of HPV 43 and HPV 44 was done by SBH and both HPVs were most closely related to types 6 and 11. HPV 43 and HPV 44 together were found in 6 of 439 normal cervical tissues, in 80 of 195 CINs, but in none of 56 ICCs tested. Thus, HPV 43 and HPV 44 are relatively uncommon HPV types associated with low-grade lesions. (Lorincz et al 1989).

2.3.d. Transmission of HPVs

HPV infections are usually transmitted by direct skin and mucosal contact. HPV gains entry through microabrasions and enters basal cells by direct binding to cell surface ligands. The specific receptor has not been identified, although there is evidence to suggest that both the α 6-integrin family and heparan sulphate may play a part (Giroglou et al 2001, Evander et al 1997). In response to infection, cells in the basal layer of the epithelium proliferate rapidly to form the wart. All the cell layers of a wart correspond to normal differentiated epithelium and histologically, the infected granular layer appears to contain vacuolated cells (koilocytes) with nuclear abnormalities.

Among the sexually transmitted viruses, HPVs are the most common, infecting both men and women (Hildesheim et al 1994, Ley et al 1991). The age curve of cervical HPV prevalence shows a peak at age 16-25 years, suggests that the transmission of HPV infection to the cervix often occurs soon after the initiation of sexual intercourse. Also the prevalence of cervical HPV infection declines sharply with age (Schiffman 1992). The cumulative lifetime probability of acquiring a cervical infection with at least one type of HPV is extremely high for sexually active individuals (Schiffman 1992).

2.3.e. Mechanism of carcinogenesis

Persistence and integration of HPV DNA

Even with infection by one or more common oncogenic HPV, not all women develop ICC suggest that additional events are necessary. It has been claimed that one important factor for the later progression is the ability of the infection to persist and continuous expression of viral genes over a long period of time, regardless of whether the infection is manifested clinically or subclinically. Nobbenhuis et al (1999) and Kjaer et al (2002) showed that persistent infection with HRHPV is necessary for development and maintenance of CIN III. Other study suggested that HPV persistence is an important event for the evolution and maintenance of cervical cancer (Milde-Langosch et al 1995). Chua and Hjerpe (1996) demonstrated the persistence of HPV DNA in the cervix by showing the presence of HPV DNA in archival smears many years before ICC was

diagnosed. HPV DNA was demonstrated in 67% of the smears (PCR) that were taken 1.5 to 7 years before a ICC was diagnosed, whereas the virus was found in only 11% of the matched control samples. Factors that may predispose to persistence of oncogenic HPV 16 or 18 infection may include estrogens or progestins (Thomas et al 2001).

The integration of the viral DNA into the human genome is considered to be another important event in tumor progression and integration is thought to have effects on viral gene expression and the biologic behavior of HPV infected cells. As a result of integration, the viral genome persists in the cell lineage. Yee et al (1985) examined a series of human carcinoma cell lines for HPV DNA sequences with the use of HPV 6, HPV 11, HPV 16 and HPV 18 DNA probes in NCI of Bethesta, Maryland. Six of the eight human ICC cell lines examined in this study contained integrated HPV DNA sequences (detected either with an HPV 16 probe or with an HPV 18 DNA probe). This information also support that integration of HPV DNA with the cervical cell DNA has a role in the mechanism of carcinogenesis.

Several other studies also demonstrated the integration of viral sequences in the host-cell genome in genital cancers (Cooper et al 1991, Meanwell et al 1987, Di Luca et al 1986). Popescu and DiPaolo (1990) for the first time revealed in an experimental system that HPV 16 integration into the cellular genome was associated with the induction of a subset of chromosome alterations. HPV16 integration frequently occurred at fragile sites and near proto-oncogenes may be a critical alteration which confers a selective growth advantage and an indefinite proliferative potential to HPV-transfected cells.

In benign cervical precursor lesions, viral DNA is maintained as a free, extrachromosomal, circular form termed as "episome" and in ICC the viral DNA appeared to be integrated within the host genome in most of the cases (Durst et al 1985). In cell lines derived from ICC and human keratinocytes transformed in vitro with HPV, the HPV DNA is integrated into the host genome. Episomal forms of the virus was detected in samples of inflammatory states and CIN grade I lesions. In invasive tumours and in CIN III lesions there were no episomes detected, suggesting that lesions with integrated HPV 16 precede the invasive stage (Daniel et al 1995).

Cullen et al (1991) reported that integration is not always required for malignant progression. Integration was detected in 72% of the HPV 16 associated carcinoma compared 100% of the HPV 18 associated carcinoma. Moreover in some cases both integrated and episomal form of HPV DNA were detected in the same tissue sample.

In human and animals, two proteins called Retinoblastoma (Rb) gene and p53, regulate cell division. Protein products of HPV early genes (E6, E7) have been identified that interact with these growth-regulatory proteins (p53, Rb) of the human cell and provide a likely mechanism for an HPV oncogenic effect. p53 is a cell growth regulatory gene and two mechanisms are involved in the p53 suppressor gene inactivation which is associated with the etiology of cervical neoplasia. One mechanism involves binding of oncogenic HPV E6 protein to the p53 protein and inactivation of this gene by degradation (Scheffner et al 1990). Due to the inactivation of p53, its tumor suppressor activity is hampered. Panotopoulou et al (1997) reported that HPV 16 and 18 encode transforming proteins, capable of binding and forming complexes with p53 protein. This is the common mechanism of p53 inactivation as oncogenic HPV subtypes have been shown to be present in about 90% of cervical carcinoma tissue samples (Bosch et al 1995).

Another mechanism of inactivation of the p53 protein product suppressor activity is mutations in this gene. This is an infrequent mechanism of p53 gene inactivation (Busby-Earle et al 1993, Fujita et al 1992).

Chromosomal aberrations

Chromosomal aberrations are known to be related to different biological behaviors of malignant lesions. A crucial role of HPV in cervical carcinogenesis through changes in the chromosomal pattern was shown by Christine Main et al (1999). Their results showed a highly significant correlation between numerical chromosomal aberrations and HPV positivity, which suggested that HPV DNA integration in human DNA may induce these multiple genetic alterations considered to be the cause of carcinogenesis.

2.4 Other factors and events related to cervical carcinogenesis

A high percentage of young women are infected with one or more HPV types during their reproductive years and only a few develop ICC. Other co-carcinogens, the immune status of the individual, nutrition and many other factors may influence for the persistence of HPV infection as subclinical (latent) state and turning into a precancer, or eventually to cancer. The mechanisms by which HPV infection and other risk factors interact in the carcinogenesis have not been well-described by sufficient population-based studies. The prolonged interval between the beginning of HPV infection and cancer development indicates that HPV infection alone is insufficient for cervical carcinogenesis.

Numerous and diverge risk factors have been noted to have influence on development of ICC including increasing age, socioeconomic variables, reproductive status, sexual behaviour (early age at first intercourse, greater number of sexual partners), use of contraceptive methods and health related factors (smoking, diet and nutrition).

Initiation of sexual activity at an early age is associated to the risk of cervical cancer. Wynder et al (1954) clearly shown that ages at marriage of ICC patients were lower than those of control patients and this effect persisted even after elimination of effect of number of marriage (Wynder et al 1954). The association of cervical neoplasia with sexual behaviour has created the interest to explore it's relation with STAs. The estimation of the role of any STA in cervical oncogenesis is difficult as it is hard to individualize those STAs that have aetiological importance for cervical neoplasia from those that are indicators of sexual activity. Some of these STAs as HSV II, *Neisseria gonorrhoeae, Chlamidia Tracomatis* and *Treponema Pallidum* may act as cofactors.

2.5 Laboratory diagnosis of HPV infection in cervical sample

Laboratory diagnosis of HPV skin infection is not usually necessary as most papillomas are benign and can be recognised clinically. But in case of genital tract infection, the situation is different due to it's association with cancer. HPV can not be diagnosed by isolation in routine monolayer cell culture as the production of infectious viral particles requires a fully differentiated epithelium. The lack of a reliable serological test for HPV

detection and the inability to cultivate HPV in vitro has necessitated the diagnosis of this virus by molecular techniques. A variety of nucleic acid hybridisation assays have been developed for the detection of HPV infection from samples obtained with swabs, scrapes, brushings, lavages and biopsy specimens. Recently attention has been focused for HPV tests to determine the HRHPV types in a specimen.

There are two categories of HPV DNA detection methods used in population studies (Lorincz 1992):

Methods - Identification of the nucleic acids directly

It includes SBH, dot blot hybridization (e.g. ViraPap and Profile kits, Digene Diagnostics, Silver Spring), ISH and HC liquid hybridization (Digene Diagnostics).

In SBH, DNA is extracted from the specimen and the DNA preparation is digested with one or more restriction enzymes (restriction endonuclease). The resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured and transferred from the gel to a solid support (usually a nitrocellulose filter or nylon membrane) to which they bind strongly. The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to radiolabeled DNA or RNA, and autoradiography is used to locate the positions of bands complementary to the probe.

The dot blot hybridization is a simplified Southern blot method. In this method after digestion and denaturing of DNA, the specimen was spotted onto the nylon filter, hybridized with specific HPV probes (Kiviat et al 1990).

ISH is a technique by which whole chromosomes or specific portions of chromosomes can be visibly marked. This is achieved by hybridizing a labelled nucleic acid probe with target nucleic acids in cells, tissue sections or metaphase spreads. Probes can be labelled with radioactive isotopes or nonradioactive reporter molecules such as biotin, digoxigenin or 2-amino-acetylfluorene and this can be detected by immunocytochemical methods (Jourdan et al 1995). A variety of approaches to the visualization of non-isotopically labelled probes have been reported over the past few years. These include enzymatic detection with a coloured end precipitate, chemiluminescence with photobiotin

(Sano et al 1998, Lizard et al 1997, Bleiweiss et al 1992), immunocytochemical detection with colloidal gold (Cheung et al 1999), and fluorochrome conjugated antibodies by fluorosence microscopy.

ISH with non-radioactive HPV probes in paraffin-embedded histologic tissues provides a direct assessment of the relationship between HPV and morphology in histologic specimens. Because the sensitivity of the ISH technique is approximately 20-50 viruses per cell, only a positive test is of clinical value (Nuovo 1992).

HC II assay used signal amplification to detect HPV DNA. HC II detects 13 HRHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and is standardized and highly reproducible. A commercial kit that detect HRHPV types by HC II (Digene corporation) has been approved by United States FDA. The HC II test involves a laboratory process that produces light signals roughly proportional to the amount of HPV DNA present in the specimen. A Zimbabwe study found that in screening for HSIL, the test sensitivity was 81% and specificity was 62%. Sensitivity and specificity for LSIL were 64% and 65%, respectively (Womack 2000).

Methods - Amplification of nucleic acids and detection of the product

The amplification methods used for HPV epidemiology are PCR based techniques (Young et al 1989). The PCR is an *in vitro* method for primer directed enzymatic amplification of specific target DNA sequences and has wide application. It produces highly concentrated samples of a specific DNA sequence. The samples are then probed to identify which specific HPV genotypes are present. The PCR amplify the target DNA until sufficient quantities are present to allow visualization on agarose gel electrophoresis of the ethidium-bromide-stained products. The procedure is usually carried out in an automated thermal cycler, a number of which are commercially available.

2.6 Laboratory diagnosis of *N. gonorrhoeae* in cervical sample

Culture for *N. gonorrhoeae* has long been considered as the diagnostic test of choice. Unfortunately, culture sensitivity is lower when specimens have to be transported to the laboratory from the collection site. Therefore gonococcal DNA detection tests are more appropriate for diagnosing gonococcal infection. HC II test was used for the detection of *N. gonorrhoeae* in the collected cervical sample. The test is at least as sensitive as culture for gonococci (Schaehter et al 1999).

2.7 Methodological issues related to laboratory techniques

A study of more than 9,000 sexually active women, age 18 and older, in Costa Rica found that the HC II test had a sensitivity of 88% and specificity of 89% in detecting HSIL and cancers. When results were calculated by age, specificity was highest (93.2%) for women age 41 and older. Overall, HPV testing using the HC II test was more sensitive than conventional Pap testing (88 vs. 78%) for detection of HSIL and ICCs, but less specific (89 vs. 94%) (Schiffman et al 2000). The process requires basic laboratory supplies to technologically advanced equipment. These requirements currently make the use of HC II, too costly and difficult to employ, in some low-resource settings.

Lie et al (1997) concluded that light microscope (LM) is an nonspecific method of identifying HPV infections and LM identification of HPV has no clinical implications. ISH is a costly and time-consuming method compared to PCR. They compared performances of LM, ISH and PCR and showed that PCR was a superior method. However PCR is a good method for research and not a suitable method to use on a large scale population. The considerable high skills, equipment, and costs involved, however, generally make PCR inappropriate for large screening programs in low-resource settings.

In general, PCR-based tests yield HPV population prevalence estimates about 2-3 times higher than those of nonamplified tests (Lie et al 1997). However, if ample specimen is tested, the detection of HPV DNA in prevalent cases of CIN or cancer is similar regardless of whether non-amplified of amplified tests are used, because viral load is typically much higher in case patients than in infected but cytologically normal control subjects. The PCR has potential for use in the detection of small amounts of HPV viral

nucleic acids present in clinical specimens. Primers corresponding to highly conserved HPV sequences may be useful for detecting low amounts of known HPV DNA as well as new HPV types. It can easily detect 0.01 pg of amplified DNA fragment (Gregoire et al 1989). The improved detection rate of HPV DNA by PCR was revealed by significantly greater HPV detection by this method (90.3%) than by SBH (57.1%) (Labropoulou et al 1996, Chang 1995). This variation of detection of HPV prevalence between these two existing detection methods should be kept in mind during reviewing various epidemiological data concerning HPV detection. But Riou et al showed a similar detection rate (84%) of HPV DNA sequences by both SBH and PCR in early stage ICC of the French and Algerian women (Riou 1990). This may be related to the use of more DNA probes during SBH in that study than the previous studies.

Cytology based screening is a widely used method for cervical cancer screening with good results. However, it's results can be greatly improved if complemented by a DNA test of the virus. New guidelines for cervical-cancer screening strategies in conjunction with HPV DNA test has been tested. Cuzick et al (1995) showed that HPV DNA test (PCR) can usefully augment but not replace conventional cytology and he suggested a strategy by combining cervical smears with testing for HPV.

Molecular technologies accomplished a substantial development in the recent decades and is playing significant role by better understanding of the association between HPV infections and development of cervical neoplasia. The sensitivity of commercially available HPV tests has improved a lot to detect clinically significant disease. The results of some long-term prospective studies on large numbers of females have convinced the clinicians and health care providers to be more inclined to arrange HPV DNA testing. Recent studies have examined the role of HPV DNA test as a primary screening test for cervical cancer (Schiffman et al 2000, Wright et al 2000). Data proposed the inherent value for using HPV testing as a primary screening strategy in older women. Specificity was highest (94%) in women aged 41 and older (Schiffman et al. 2000).

Use of a screening test depends on the achievable levels of sensitivity and specificity. The great attraction of HPV testing is its high sensitivity. Several studies have shown sensitivities of approximately 90% or greater for the second-generation HPV test (Clavel

et al1999, Cuzick et al 1999). Comparative studies indicate a higher sensitivity than that achieved by cytology (Clavel et al 1999, Cuzick et al 1999, Reid et al 1991). The sensitivity of HC-II is quite similar to that of PCR using 'consensus' primers in previous studies (Bosch et al, 1995, Van den Brule et al 1991).

Many controversial issues have been published on different aspects like clinical and sociodemographic concepts regarding aetiology of cervical cancer. From the findings of this extensive literature review, it was revealed that though plenty of study have been carried out, adequate data on case control study is lacking on this subject in this subcontinent. So it was necessary to explore on this unexplored area to gather information which ultimately may be very useful both nationally and internationally. In the view of these observations study on "Association of Human Pappilomavirus with cervical intraepithelial neoplasia and cancer in Bangladeshi women" seems to be important to see facts according to sociodemographic status of this country.

CHAPTER 3:

AIMS AND OBJECTIVES

CHAPTER 3

AIMS AND OBJECTIVES

The aims and objectives of the present study were as follows:

3.1 Aims and objectives

3.1. a. Aims

The study is aimed to determine the association of HPV infection and its risk group with cervical intraepithelial neoplasia and cervical cancer among Bangladeshi women, to evaluate the association of socio-economic and demographic factors with cervical cancer.

3.1. b. Objectives

To fulfil the aims following objectives needed to be achieved:

i) To determine the prevalence of HPV infection and its risk group among a segment of married women population with CIN, cervical cancer and normal cervix.

ii) To assess the association of different risk group (high risk and low risk) of HPV infection on progression of the neoplastic changes.

iii) To determine the association of socio-economic factors (education, occupation, monthly income) on prevalence of CIN, cervical cancer and HPV infection (high risk and low risk).

iv) To determine the association of demographic factors (sexual, reproductive and contraceptive) on prevalence of CIN, cervical cancer and HPV infection (high-risk and low-risk).

3.2 Hypotheses:

Based on the facts mentioned, in the present study it has been suggested that HPV may play an etiologic role in developing cervical cancer. However, the following hypothesis were proposed to be tested :

- High and Intermediate risk HPV (HRHPV) infection has central role in developing CIN and cervical cancer
- Low economic condition has an association with developing cervical cancer
- Low education level has an association with developing cervical cancer
- Early age of 1st marriage and 1st intercourse increase the risk of developing cervical cancer
- Early age of 1st child birth increases the risk of cervical cancer
- Multiple marriage of both women and husband increases the risk of cervical cancer
- High parity and total number of pregnancy increases the risk of cervical cancer
- Oral contraceptive pills may increases the risk of cervical cancer
- Some sexually transmitted diseases (STDs) as syphillis, gonorrhoea and HSV II may have association with cervical cancer

CHAPTER 4

MATERIALS AND METHODS

CHAPTER 4

MATERIALS AND METHODS

4.1 General outline

This case-control hospital based study was carried out to find out the association of HPV and its risk-types with CIN and ICC. Subjects for the study were recruited from Bangabandhu Sheikh Mujib Medical University (BSMMU) and Cancer Institute and Research Hospital (CIRH) of Bangladesh between October 1999 and May 2002. This research work was approved by the Bangladesh Medical Research Council-(BMRC) Ethical Review Committee. The women were invited to participate in the study before the procedures were undertaken. The procedure was explained, and women who agreed to participate provided written informed consent. The study was carried out on women with ICC (N=123), CIN (N=70) and normal cervix (N=120). Age was matched among the ICC and control group.

Data were collected after counseling and motivating the study population. Each woman was inquired about epidemiological factors, factors associated with cancer cervix and HPV infection (questionnaire in page 123-124). General, abdominal and vaginal examinations were carried out and the findings were noted in the same questionnaire. Cervical samples and samples of serum were collected from all three groups of women and preserved. In all patients, cervical samples obtained were tested for the HPV DNA using the HC II test. This assay is capable to demarcate between HRHPV and LRHPV types. *N. gonorrhoea* DNA was tested from the samples of CIN and control group using the HC II. Serum of all three groups of women were tested for *T. Pallidum* and HSV II immunoglobin G (IgG). Cytological, virological and immunological investigations were conducted in the respective departments of BSMMU and Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Diseases (BIRDEM).

4.2 Selection of subjects

4.2.a. Description of the study area

This research was conducted in the Department of Obstetrics and Gynaecology, Pathology and Virology of BSMMU and Department of Immunology of BIRDEM. Subjects for the study were recruited from BSMMU and CIRH, Dhaka between October 1999 and May 2002. The Department of Obstetrics and Gynaecology, Pathology and Virology are integrated part BSMMU. BSMMU formerly known as Institute of Postgraduate Medicine and Research (IPGMR), started its activity as referral postgraduate institute and hospital in 1965 to promote medical education and research including different postgraduate courses. IPGMR was converted to BSMMU in April, 1998. Out of total 800 beds, the Department of Obstetrics and Gynaecology has 70 beds. The department runs a colposcopy clinic dedicated for cervical cancer screening. Along with management of CIN patients about 150–200 gynaecological cancer cases have been recorded and managed each year.

CIRH of Bangladesh is a tertiary level institute involved in management of all types of cancer patients from different corners of the country.

Part of the laboratory investigations were conducted in the Departments of Immunology of BIRDEM which is also a tertiary level institute. The Department of Immunology is running M.D., and M.Phill courses since 1997. Apart from identification of immunological diseases it has facilities related to tumour biology, immunochemistry, immunogenetics, cytogenetics and molecular diagnostic facilities (PCR, ISH, HC II).

4.2.b. Study population selection

Non-pregnant volunteers with history of sexual activity (married, separated, divorced or widowed) between 22 to 70 years were recruited. In this study, only Bangladeshi women were recruited and none from the ethnic minority groups were included. Distribution of the study population was assessed according to their living status in the 'urban' or 'rural' areas where they spent majority of their life time. The characteristics of 'Urban area' has been described in the appendix (page 125).

4.2.c. Sample size

The sample size for this study was 384 by following the standard sample size estimation formula. Since the prevalence of HPV infection in Bangladeshi women is not known, the minimum sample size can be determined by the following formula:

$$N = \frac{Z^2pq}{d^2}$$

Where,

N = The desired sample size

Z = The standard normal deviate, usually set at 1.96 which corresponds to the 95% confidence level

p = The proportion in the target population estimated to have a particular characteristic. As there is no known estimate, then 50% can be used.

q= 1.0 - p. d = Degree of accuracy desired, usually set at .05

$$N = \frac{(1\ .96)^2\ (.50)\ (.50)}{(.50)^2}$$

= 384 women required.

Considering the fund constraint and other limitations a multiphase sampling were done. Accordingly, out of 384 cases, 314 cases were included in this study. These 311 women were divided into three groups (women with histologically diagnosed CIN, ICC and women with normal cervix as control).

4.2.d. Selection criteria

Invasive cervical carcinoma (ICC)

Data were collected from 123 women with histologically diagnosed cases of ICC attending the gynaecology outpatient department (OPD) of BSMMU (N= 58) and CIRH (N= 65). The stage of the disease was assessed clinically according to International Federation of Gynecology and Obstetrics (FIGO) criteria and it has been described in the appendix (page 125).

Cervical intraepithelial neoplasia (CIN)

In the gynaecology OPD of BSMMU collection of cervical smear is routinely offered to all women and the cases of abnormal smears had colposcopical examination (appendix page 125) in the same institute. Seventy study subjects of CIN group were enrolled from the women of histologically diagnosed cases of CIN and patients of all three grades of CIN (CIN 1, CIN II, CIN III) were recruited.

Normal cervix (control)

Another 121 women with normal cervical smear and normal colposcope findings were randomly recruited as control group from the women attending the gynaecology OPD BSMMU after matching of age with the ICC group. They were recruited from the women attending for gynaecological check-up, ovarian tumour, myoma of uterus and hysterectomy for different indications (other than malignancy) and other gynaecological problems. The cases with history of vaginal discharge and cervicitis were not recruited in this group.

4.3 Laboratory methods

4.3.a. Histological record

The biopsies were paraffin-embedded and HE-stained sections were analyzed according to routine procedures. The histological diagnoses distinguished between normal epithelium, showing disturbed maturation without atypia, CIN I, CIN, CIN III and ICC. The Department of Pathology of BSMMU supported by carrying out the cytological and histopathological examinations and it's quality was controlled by continuous supervision by senior persons.

4.3.b. Sample collection

Cervical sample collection and storage

Cervical Sampler (Appendix page 126, Fig. 4.1) was used for collection and transport of cervical specimens of all three groups. DNA specimen was collected prior to application of acetic acid or iodine if a colposcopy was planned at the same time. Excess mucus was removed from the cervical os and surrounding ectocervix using cotton swab. The brush

was inserted 1-1.5 cm into the cervical os until the largest outer bristles of the brush touched the ectocervix. The brush was rotated three full turns in a counter clockwise direction. Care was taken so that the brush was not completely inserted into the cervical canal. The specimens were stored at -20°C upon receipt at the laboratory and tested within three months in batches.

Blood collection and storage

A sample of 5 ml of whole blood was collected from each subject by venipuncture and the collected blood was transported to virology laboratory of BSMMU within one hour of collection. The blood containing tubes were allowed to remain at room temperature for one or two hours on the bench. The clot was detached from the tube wall by pasteur pipette tip and all clot free liquid or serum was sucked out by the same pipette and aliquoted into a microcentrifuge tube (roughly 1.0 ml serum/1.5 ml tube). Centrifugation at 1500 rpm for five minutes was done prior to serum collection. Samples were stored in – 20°C freezer and a generator was available to run the freezer during power breakdowns.

4.4 HPV DNA determination

The Department of Immunology of BIRDEM provided the laboratory set-up and necessary support to carry out HC II for detection of HPV DNA of the samples collected from cervix of CIN, ICC and control group.

The Digene HPV Test using HC II technology is a linear signal amplified hybridization antibody capture microplate assay. Qualitative detection of eighteen types of HPV DNA in cervical specimens was carried out using chemiluminescent detection. The Digene HPV Test can differentiate between two HPV DNA groups:

Low risk HPV types - 6/11/42/43/44

High/ intermediate risk HPV types - 16/18/31/33/35/39/ 45/51/52/56/58/59/68.

4.4.a. Principle of the procedure

Specimens containing the target DNA hybridize with a specific HPV ribonucleic acid (RNA) probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with polyclonal universal antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for RNA:DNA hybrids, and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units (RLUs) on a luminometer (page 126). The intensity of the light emitted denotes the presence or absence of target DNA in the specimen.

i. Denaturation of Specimen

The specimen was treated with NaOH (Provided in kit) and mixed. The reaction was then incubated at 65°C for 45 minutes in water bath to denature the nucleic acid and to convert it as single strand target for the hybridisation step.

ii. Hybridisation (with RNA probe)

A target specific RNA probe cocktail from the kit was added to the microtube and mixed for two to three minutes on the rotary shaker. It was then incubated for 60 minutes in water bath at 65^0C to complete the hybridisation reaction.

iii. Transfer and capture hybrids

The hybridised sample was transferred to a microplate coated with a universal polyclonal Anti RNA:DNA antibody. This microplate was incubated for 60 minutes at room temperature (25^0C) on a rotary shaker to capture the RNA:DNA hybrid. There was no binding towards single stranded RNA or DNA which get eliminated by aspirating untreated solution.

iv. Conjugation and washing

Immobilised hybrids were reacted with alkaline phosphatase conjugated antibody specific for RNA:DNA hybrids at room temperature (25^0C) for 30 minutes. All

unreacted probes were washed away reducing non-specific binding. The microplate was washed with buffer to remove unbound conjugate.

v. Detection of hybrids with chemiluminescence

CDP-Star with Emerald II, a chemiluminescent substrate, was added to the microplate and incubated at room temperature (25^0C) for 15 minutes. The microplate was then read in the DML 2000 luminometer and the RLUs were measured and compared to the standard using the Digene system software.

Cut off value

An RLU measurement equal to or greater than the cut off value indicates the presence of target HPV DNA sequences in the specimen. An RLU measurement less than the cut off value indicates the absence of the specific HPV DNA sequences tested or HPV DNA levels below the detection limit of the assay.

4.4.b. Test procedure

i. The specimens and all required reagents (page 126) were taken out from the refrigerator prior to beginning the assay. These were allowed to reach 20-25^0C for at least 15 to 30 minutes.

ii. The calibrators and specimens to be tested were placed in a test tube rack, in the same order in which they were planned for testing. The calibrators must be tested first. Negative Control (NC), Calibrator A (CA) and/or Calibrator B (CB) and specimens were run in an eight microwell column configuration. The data sheet provided were used to create the calibrator/specimen template.

iii. In the Combined Probe Cocktail (CPC) Method, NC, CA and CB were tested in triplicate with the CPC in the same microplate. A1, B1 and C1 wells were used for the NC and wells D1, E1, F1, G1, HI and A2 were used for CA and CB. In Low risk Probe A or High risk Probe B method performed individually, NC and CA were tested in triplicate with HPV Probe A cocktail in one microplate, and NC and CB were tested in triplicate with HPV Probe B cocktail in a second microplate. Wells

A1, B1 and C1 were used for the NC and wells D1, E1, and F1 for CA or CB as the case may be.

Table 4.1: Representative Layout for CPC Run of 24 Microwells:

Row		Column	
	1	2	3
A	NC	CB 3	Specimen 8
B	NC	Specimen 1	Specimen 9
C	NC	Specimen 2	Specimen 10
D	CA 1	Specimen 3	Specimen 11
E	CA 2	Specimen 4	Specimen 12
F	CA 3	Specimen 5	Specimen 13
G	CB 1	Specimen 6	Specimen 14
H	CB 2	Specimen 7	Specimen 15

iv. Specimens were tested once with the CPC if using the CPC method or once with HPV Probe A cocktail and once with HPV Probe B cocktail if using the Two-Probe method.

Denaturation: Combined Probe cocktail and Two-Probe methods

i. The screw caps on calibrators were loosen and caps from specimens were removed and discarded.

ii. Denaturation reagent with indicator dye were pipetted into each calibrator or specimen using a repeating adjustable pipettor. Special care was taken not to touch the sides of the tube or cross contamination of specimens. The volume of denaturation reagent needed is equivalent to half the sample volume. The exact volume for each type of calibrator and specimen was listed in the table below.

iii. The caps on the calibrators were tightened and the specimen tubes were recapped with clean screw caps.

Each tube were mixed thoroughly by vortexing individually, at high speed, for five second. There must be a visible vortex of liquid inside each tube during mixing such that the liquid washes the entire inner surface of the tube. The control, calibrators and specimens should turn purple.

The specimen tube was inverted once to wash the inside of the tube, cap and rim. The tubes were returned to rack.

Calibrator or Specimen	Vol. of Denaturation Reagent Required
Negative Control	1000µl
Calibrator A or B	500µl
Cervical Specimen	500µl

iv. The tubes along with the rack were incubated in a 65±2^{0}C water bath for 45 ±5 minutes (denatured control, calibrators and specimens were tested immediately, or stored at –20^{0}C whenever necessary).

v. CPC or HPV Probe A and HPV Probe B cocktails were prepared during this incubation. (Reagent preparation - page 127-128).

Hybridization and capture: Combined Probe cocktail and Two Probe methods

[Note: HPV Probe mixes supplied in the kit was viscous. Care was taken to ensure thorough mixing and that the required amount was completely dispensed into each microtube. (Reagent preparation - page 127-128)].

i. The required number of clean hybridization microtubes were placed into the microtube rack according to the method used:
   a) One set hybridization microtubes for the CPC method or
   b) Two sets of hybridization microtubes (one set for HPV Probe A and one set for HPV Probe B) for the Two-Probe method.

ii. 25µl of appropriate Probe mix were placed into each microtube using a repeating pipettor.

iii. The control, calibrators and specimens were removed from the water bath after completion of incubation.

iv. Each tube were vortexed individually for at least 5 seconds just prior to removing aliquots. 75µl of each calibrator or specimen were placed into the bottom of appropriate hybridization microtube following the template created under Set UP. A

clean extra-long pipette tip were used for each transfer to avoid cross-contamination of specimens or calibrators.

v. The microtubes were covered with plate sealers and shaked for 3 ±_2 minutes at 1100 ±100 rpm on a rotary shaker. The control, calibrators and specimens should turn yellow after shaking. Tubes that remain purple may not have received the proper amount of probe. An additional 25µl of probe to samples that remain purple were added and these were placed in shaker again as before.

vi. The microtube rack was incubated in a 65 $\pm 5^0$C water bath for 60 ±5 minutes.

vii. The required number of capture microplate wells were removed for the run from the preassembled plate. The unused microwells were returned to the original bag and it was resealed. With a marker, each column was numbered like 1, 2, 3 ... The samples were added to the wells according to the example layout shown on the template previously prepared under set up:

viii. The microtube rack containing control, calibrators and specimens were carefully removed from the water bath.

ix. The entire contents of the control, calibrator and specimen microtubes were transferred to the bottom of the corresponding capture microwell using an 8 channel pipettor set at 100µl. New pipette tips were used for each column transferred and each pipette tip was allowed to drain well to ensure complete sample transfer.

x. The microplate was covered with a new plate sealer and shaken on rotary shaker at 1100 ±100 rpm, at 20-25^{0C} for 60 ±5 minutes.

xi. During this incubation, wash buffer was prepared

xii. After completion of the capture step, the capture microplate was removed from the shaker and the plate sealer was carefully removed. The liquid from the wells were discarded: the plate was fully inverted over the sink and was shaken hard with a downward motion. Reinvertion of the plate was carefully avoided and it was blotted by tapping firmly 2-3 times on clean absorbent paper. Particular care was taken to ensure removal of all liquid from the wells and to make the top of the plate dry.

Hybrid detection and signal amplification

The appropriate volume of detection reagent 1 (DR 1) was kept into a reagent reservoir. 75µl of DR 1 was carefully pipetted into each well of the capture microplate using an 8 channel pipette. Verification was performed so that all wells have been filled accurately by observing the intensity of the pink color. All wells should have similar intensity. The plates was covered with clean Parafilm and incubated at 20-25^{0}C for 30 ± 3 minutes.

Washing

i. DR 1 was removed from the wells by placing clean absorbent or tissue paper on top of the plate and carefully inverting it. The plate was allowed to drain for 1-2 minutes. The plate was blotted well on clean absorbent or tissue paper.
ii. Using hand wash with squeeze bottle the plate was washed 6 times. Washing began at A1 and continues in a serpentine fashion to the right and downward. After all the wells have been filled, the liquid was discarded into sink with a strong downward motion. The second wash was started at well H12 moving in a serpentine motion to the left and upward. This sequence of two washes was repeated two more times for a total of six washes per well.
iii. After washing, the plate was blotted by inverting on clean tissue paper and tapping firmly 3-4 times. The toweling was replaced and blotted again. The plate was kept inverted and allowed to drain for 5 minutes. The plate was replaced one more time.

Signal amplification

i. 75 µl of detection reagent 2 (DR 2) was added into each well of the capture microplate using an 8 channel pipettor as previously described. All microwells turned to a yellow color. It was carefully verified that all wells have been filled accurately by observing the intensity of the color. All wells should have similar intensity.
ii. The microplate was covered with clean parafilm and incubated at 20-25^{0}C for 15 minutes. Direct sunlight was avoided.
iii. The microplate was read on the DML 2000 Luminometer (Fig 4.2) after 15 minutes of incubation (and no later than 30 minutes of incubation).

iv. In the DML 2000 Luminometer, assay specific software allowed the entry of appropriate run information directly into the spreadsheet.

v. Whenever a full microplate was not used, the used microwells were removed from the microplate holder, the holder was rinsed thoroughly with deionized water, made dry and reserved for next assay.

4.4.c. Quality control (page 128-129)

4.4.d. Limitation of the test (page 129)

4.4.e. Cut off calculation

Once an assay has been validated, the cutoff Values for determining positive specimens are as follows:

i. CPC method: $\frac{(CA\bar{X} + CB\bar{X})}{2}$

ii. Two-Probe method: Probe A cut off = $CA\bar{X}$

Probe B cut off = $CB\bar{X}$

Table 4.2: Example of cut off calculation for probe A of two-probe method:

	NC RLU Values	CA RLU Values
	97	312
	101	335
	91	307
Mean Value	96	318
%CV	4.9	4.7
$CA\bar{X}/NC\bar{X}$		3.31

Positive cut off value for Probe A ($CA\bar{X}$) = 318

All specimen RLU values were converted into a ratio to the appropriate cut off value. For example, all assays tested with HPV Probe A was expressed as specimen RLU/ cut off value A.

The same was done with specimens tested with HPV Probe B or the CPC Probe. These ratios were recorded on the data sheet provided (page 132-143).

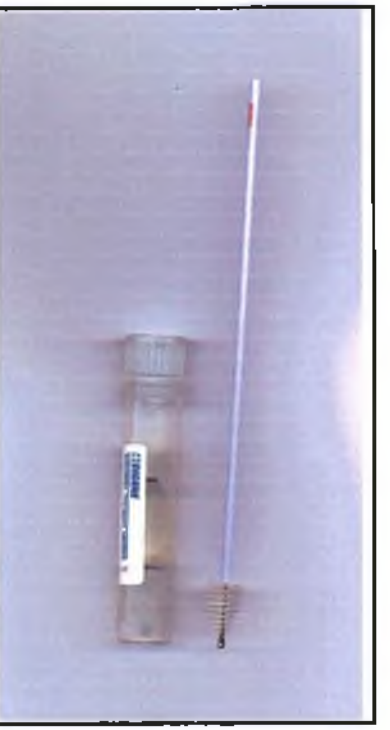


Fig 4.1 :Cervical samples are collected using a special brush and tube containing transport media

Fig 4.2 : Prepared microplate placed inside the specially prepared hardisk of the computer for detection of chemilumineseance of the DNA

4.4.f. Interpretation of specimen results

By the criteria of the Digene HPV test:

Specimens with RLU/ cut off value ratios ≥1.0 with the CPC were considered "positive" for one or more of HPV types 6,11,16,18,31,33,35,39,42, 43,44,45, 51,52,56,58 and 68 (Low Risk + High Risk).

Specimens with RLU/ cut off value ratios ≥ 1.0 with HPV Probe A only were considered "positive" for one or more HPV types 6,11,42, 43 or 44.

Specimens with RLU/ cut off value ratios ≥ 1.0 with HPV Prove B only were considered "positive" for one or more of HPV types 16,18,31,33,35,39,45,51,52,56,58,59 and 68.

Specimens with RLU/ cut off value ratios ≥ 1.0 for both HPV Probe A and HPV Probe B were considered "positive" for one or more HPV types from each group of probes.

Specimens with RLU/ cut off value ratios < 1.0 for CPC or both HPV Probe A and HPV Probe B were considered "Negative" or "None detected" for the eighteen HPV types tested. HPV DNA sequences are either absent or the HPV DNA levels are below the detection limit of the assay.

4.5 Gonococcal DNA determination

The Department of Immunology of BIRDEM provided the laboratory set-up and necessary support to carry out HC II for detection of DNA of *N. gonorrhoea* from samples collected from cervix of CIN and control group. This technology is a linear signal amplified hybridization antibody capture microplate assay using chemiluminescent detection.

4.5.a. Principle of the procedure

Specimens containing the target DNA hybridize with gonococcal RNA probe. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for RNA:DNA hybrids, and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are

conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units (RLUs) on a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen.

i. Denaturation of specimen

The specimen was already denatured during HPV DNA detection and kept in -20°C. It was taken out of the freeze to bring it in the room temperature. The reaction was then incubated at 65°C for 45 minutes in water bath to convert it as single strand target for the hybridisation step.

ii. Hybridisation (with RNA probe)

Gonococcal RNA probe (25µl) from the kit was placed into the microtube and 75µl of each denaturation specimen was added to it and mixed for two to three minutes on the rotary shaker. It was then incubated for 60 minutes in water bath at 65^0C to complete the hybridisation reaction.

iii. Transfer and capture hybrids

The hybridised sample was transferred to a microplate coated with a universal polyclonal anti RNA:DNA antibody. This microplate was incubated for 60 minutes at room temperature (25^0C) on a rotary shaker to capture the RNA:DNA hybrid. There was no binding towards single stranded RNA or DNA which get eliminated by aspirating untreated solution.

iv. Conjugation and washing

Immobilised hybrids were reacted with alkaline phosphatase conjugated antibody specific for RNA:DNA hybrids at room temperature (25^0C) for 30 minutes. All unreacted probe was washed away reducing non-specific binding. The microplate was washed with buffer to remove unbound conjugate.

v. Detection of hybrids with chemiluminescence

CDP-Star with Emerald II, a chemiluminescent substrate, was added to the microplate and incubated at room temperature (25^0C) for 15 minutes. The microplate was then

read in the DML 2000 luminometer and the RLUs were measured and compared to the standard using the Digene system software.

Cut off value

An RLU measurement equal to or greater than the cut off value indicates the presence of target gonococcal DNA sequences in the specimen. An RLU measurement less than the cut off value indicates the absence of the specific gonococcal DNA sequences tested or gonococcal DNA levels below the detection limit of the assay.

4.5.b. Test procedure

The test procedure is similar to the test procedure used for detection of HPV DNA except that gonococcal RNA probe was used instead of the HPV RNA probe.

4.5.c. Quality control : Similar to quality control of HPV DNA test

4.5.d. Cut off calculation : Similar to cut off calculation of HPV DNA detection

4.5.e. Interpretation of specimen results

By the criteria of the Digene *N. gonorrhoea* DNA Test:

i. Specimens with RLU/ cut off value ratios ≥1.0 were considered "positive" for gonococus
ii. Specimens with RLU/ cut off value ratios < 1.0 were considered "Negative" or "None detected" for the *N. gonorrhoea* DNA.

4.6 Serological detection of *T pallidum*

The department of Virology of BSMMU provided the laboratory supports for serological tests for *T pallidum*. Antibody to *T pallidum* was tested by a specific, sensitive passive haemagglutination test known as treponema pallidum haemaglutination (TPHA) test (OMEGA Corporation of UK).

4.6.a. Principle of the procedure

Rengents consists of *T. pallidum* antigen coated formolised tanned fowl erythrocytes (sencitised cells), uncoated formolised tanned fowl erythrocytes (control cells), diluted buffer, positive and negative control sera. When diluted positive sera are mixed with sensitized erythrocytes, antibody to the *T. pallidum* antigen causes agglutination of the cells. The cells form a characteristic pattern at the bottom of a microtitration plate well and the control cells form a compact button in the well. Control positive and negative sera should show positive and negative reaction for validation of test.

Procedure : (page 130-131)

4.6.b. Results and Interpretation

The titre is the highest dilution showing agglutination. The reactive control serum should produce a titre within one doubling dilution of 1/2560. The starting dilution for the quantitative procedure is 1/80.

4.7 Serological detection of HSV II

The Department of Virology of BSMMU provided the laboratory supports for serological tests for HSV II. HSV II IgG antibodies in the serum of the study population were detected by an enzyme linked immunosorbent assay (ELISA). The kits for detection of HSV II IgG antibodies were supplied by OMEGA Corporation of UK.

Principle of the Assay

Specific, inactivated, HSV II antigens are coated onto microtitration wells. Test sera diluted 1/20 are applied.

Specific antibodies to the HSV II antigens bind to the antigen in the wells. Unbound material is washed away and anti-human IgG, conjugated to Horseradish Peroxidase, is applied. The Conjugate binds to the human antibodies bound to the antigen. Unbound material is again washed away.

On addition of the substrate (Tetra methyl benzidine), a colour develops only in the wells containing enzyme, indicating the presence of human anti-HSV II antibody. The enzyme

reaction is stopped by the addition of stop solution and the absorbance is then measured at 450 nm. The concentration of specific IgG antibody is directly proportional to the colour intensity of the test sample.

Assay procedure, calculation and interpretation of results, quantitative calculation of results are described in page 131-132.

4.8 Data collection and statistical analysis:

Study subjects participated in standardized, structured interviews through a pre-tested questionnaire. The study questionnaire (appendix page 123-124) included parameters of personal history along with clinical information, socioeconomic status, lifetime sexual behavior, reproductive history, use of contraceptive methods and other demographic data. Along with the socioeconomic factors data were collected regarding education of women, occupation of women and husband and monthly income of the family (monthly salary of the earning members and monthly household income if there was any). The women were asked regarding her husband's nature of job that can identify the travelling and stay away from family including duration of stay away from home and frequency of their travelling.

A detailed history regarding use of different methods of contraception was collected. Women continued a particular contraceptive method for at least one year were considered as user of that particular method. Duration of use of each method of contraception was noted separately.

Specimens from the cervix were collected for all three groups. The questionnaire also collected information about laboratory report forms.

All interview and data were collected either by the researcher herself or under her direct supervision by well trained research assistance. A good liaison was kept with the laboratories involved. Professor Shahla Khatun, the supervisor, and two co-supervisors Professor Farida Huq and Professor M Nazrul Islam provided continuous guidance. All laboratory works and it's quality control were supervised by senior persons.

4.8.a. Variables:

Independent variables:

Age, education of couple, occupation of couple, income, age of marriage, number of marriage of couple, duration of sexual activity, husbands travelling status, age at first delivery, parity, total number of pregnancy, use of different contraceptive methods, report of HPV and gonococcal DNA by HC II, reports of seroprevalence of HSV II and *T. pallidum*.

Dependent variables:

Cervical cytology report, colposcopy findings and histopathology report of cervical tissue of all three groups of women.

4.8.b. Statistical analysis

Completed interviews were reviewed, edited and processed for computer data entry. The data were entered and the data analysis were performed using Statistical Package for the Social Sciences (SPSS) program software. Initially univariate analysis was used to observe the frequencies, mean and standard deviation (SD) of different variables. Later on the association of HPV infection with CIN and ICC and different socio-demographic factors were analysed by bivariate analysis and statistical tests (χ^2 and unpaired t test).

Logistic regression was used to test the statistical significance of interactions. The relative risk (RR) as estimated by the odds ratio (OR) was the measure of association used for evaluating effects of HPV and different socio-demographic and reproductive factors. Unconditional logistic regression analysis was used for potential confounding variables and to observe the independent influence of some of the factors, to get maximum likelihood estimates of RR and to assess OR along with 95% confidence interval (CI).

4.9 Ethical considerations

Ethical approval from BMRC was obtained through submission of required documents of the research protocol. The sensitive issues of the research was taken into account during data collection. Good rapport among the accompanying person as well as individual respondents was kept for successful completion of the research. Proper care was taken to the women who needed further management. All information collected were kept strictly confidential. Respondents were provided with comprehensive information regarding the procedures including their right to refuse to participate if they wish. Proper counseling was provided if respondent was found to be a positive case for the screening to avoid unnecessary anxiety and tension.

Patients were examined along with maintenance of privacy and there was no risk of social and physical hazard or psychological trauma to the women. Cervical sample collection and colposcopy guided cervical biopsy collection by punch biopsy forceps does not need anaesthesia and there was no added risk of excess bleeding. The place of study has adequate facilities for management and follow-up of CIN, cervical cancer and other gynaecological problems. Counseling of these women made them conscious about screening of cervical cancer and reproductive health problems which has important social impact.

The patient got more scrupulous check-up and opportunity of having the cytological, gynaecological and immunological examination. HC II tests were performed at Immunology department of BIRDEM which is well equipped for this investigation.

Chapter 5: Results

5.1 Human Papillomavirus

5.2 Other Sexually Transmitted Agents

5.3 Socioeconomic Factors

5.4 Sexual behaviour

5.5 Reproductive Status

5.6 Contraceptive Practice

CHAPTER 5

RESULTS

This case control study enrolled a total 123 cases of ICC. Among these cases 58 were collected from BSMMU and 65 from CIRH. Among the 123 ICC, three cases were excluded due to inadequate serum collection. Seventy CIN cases and 121 cases of the control group were recruited from BSMMU. Among the 70 women with CIN, 34 (48.6%) had CIN I and 36 (51.40%) had CIN II or CIN III.

Among the ICC group of 120, 94.2% tumours were invasive squamous cell carcinomas, 5% were adenocarcinomas and 0.8% was adenosquamous cell carcinomas. Among these, 22 (18.3%) were well-differentiated, 82 (68.3%) were moderately differentiated and 16 (13.3%) were poorly differentiated. Fourteen (11.7%) patients had stage-Ib, 33 (27.5%) stage-IIa, 42 (35%) stage-IIb, 28 (23.3%) stage-III, and two (1.7%) had stage-IV disease.

Distribution of the Study Population

Among the 120 ICC patients 67 (55.8%) came from rural areas and 53 (44.2%) from urban areas of the country (Fig 5.1). During recruitment of the women of control group, attempt was taken to maintain the similar distribution of residence as the ICC group.

Age at Diagnosis

The mean age (Mean±SD) of the women with ICC, CIN and control was 47.2±11.2, 34.9±8.2 and 45.4±10.7 years respectively. Age distribution between cases and controls were matched (p= 0.09). Six (5%) of the 120 ICC patients were younger than 30 years and the youngest patient was only 25 years of age. Among the ICC group, 25 (20%) presented before 40 years of age and 95 (80%) of them presented after 40 (Fig 5.2). In contrast to that 44 (62.8%) of the women with CIN presented before 40 years age (Table 5.1).

Table 5.1: Age Distribution

Age Distribution	Cancer (N=120)	CIN (N=70)	Control (N=121)
15- 29	06 (05.0)	18 (25.7)	09(7.4)
30-39	19 (15.8)	26 (37.1)	25 (20.7)
40-49	42 (35.0)	22 (31.4)	43 (35.5)
50 and above	53 (44.2)	04 (5.7)	44 (36.3)
Mean age	47.9± 11.0	34.9 ± 8.2	45.45 ±10.7

Cancer vs control - t=1.72, p=.09, CIN vs control - t= 7.15, p= 0.000 (Percentages are within parenthesis)

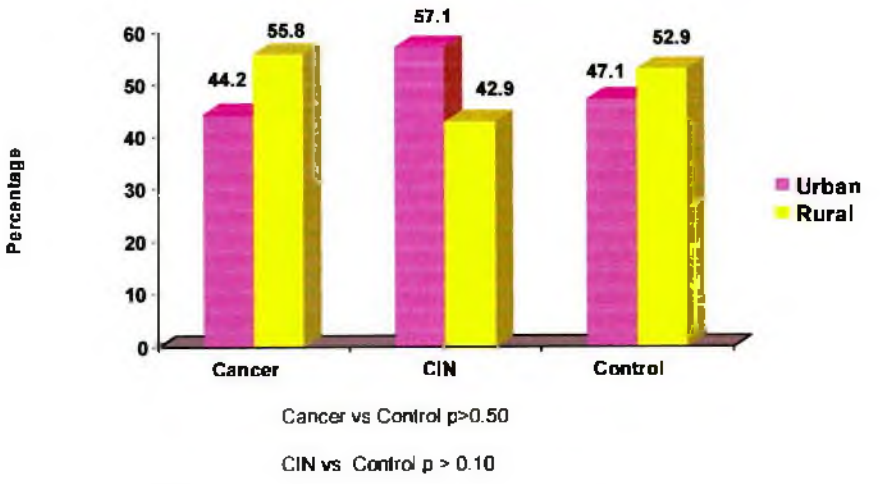


Fig 5. 1 : Distribution of living status according to urban and rural areas

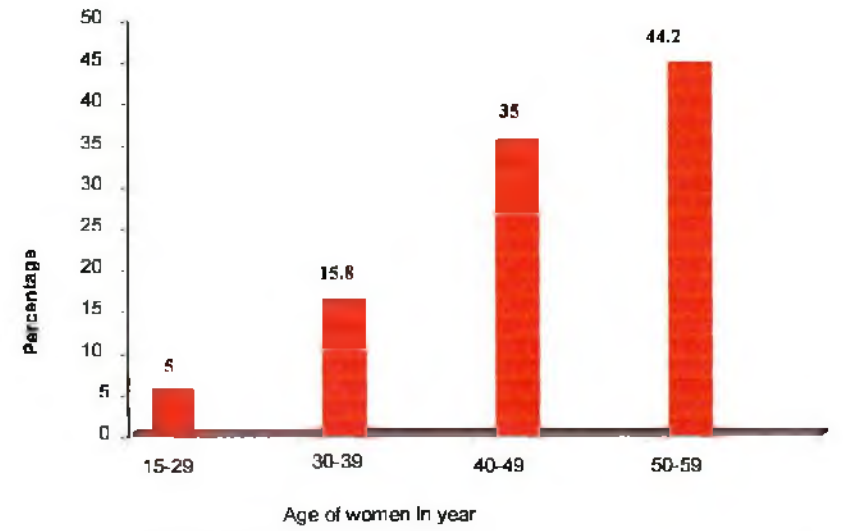


Fig 5.2 : Distribution of age of women with cervical cancer showing about 80% of women with ICC presented after 40 years of age

Among the independent variables considered in this study, genital tract infections included HRHPV, LRHPV, HSV II, *T. pallidum* and *N. gonorrhoea*. Other variables were categorized as socioeconomic factors, sexual behaviour, reproductive status and contraceptive practice.

5.1 Association of HPV with ICC and CIN

Some of the computer result sheets of the DNA detection reports of both risk groups of HPV and *N. gonorrhoea* were presented in the appendix (page 132-144).

5.1.a. Influence of HRHPV on CIN and ICC

HRHPV DNA was detected in 96.7 % of ICC, 60.0% of different grades of CIN and 4.1% of control women (Table 5.2). On the contrary, LRHPV DNA was found in 10.8% of all ICC, 22.9% of different grades of CIN and 1.7% of control women. Bivariate analysis showed that both high and low risk HPV had significant association with development of ICC and CIN.

Table 5.2 : Prevalence of High and Low risk HPV

Type of HPV	Existance of HPV	Cancer (N=120)	CIN (N=70)	Control (N=121)
High, & Intermediate risk HPV	Present	116 (96.7)	42 (60.0)	5 (4.1)
	Absent	4 (3.3)	28 (40.0)	116 (95.9)
Low risk HPV	Present	13 (10.8)	16 (22.9)	02 (1.7)
	Absent	107 (89.2)	54 (77.1)	119 (98.3)

High and intermediate risk
Cancer vs control - (yates correction) $p<0.001$ (χ^2=202.7, df =1)
CIN vs control - $p<0.001$ (χ^2= 74.6, df=1)
Low risk
Cancer vs control - (yates correction) $p<0.01$ (χ^2=7.2, df =1)
CIN vs control - (yates correction) $p<0.001$ (χ^2=20.9, df=1)

5.1.b. Influence of HRHPV on CIN

Among the 70 cases of CIN, 36 (51.4%) had CIN II or CIN III. Among these 36 cases of CIN II and III, 30 (83.3%) were HRHPV positive (Table 5.3) and this prevalence was significantly higher than that of CIN I (p=.000). Five (14.7%) of CIN I and 11 (30.5%) of CIN II and III contained LRHPV and this difference was not statistically significant (p=0.097).

Table 5.3 : Comparison of HPV Types Among the CIN

Type of HPV	Existance of HPV	CIN I (N=34)	CIN II & III (N=36)	Total
High, & Intermediate risk	Present	12 (35.3)	30 (83.3)	42
	Absent	22(64.7)	6(16.7)	28
Low risk	Present	5 (14.7)	11 (30.5)	16
	Absent	29 (85.3)	25 (69.4)	54

High and Intermediate risk – CIN II & III vs CIN I $p< 0.001$ ($\chi^2=16.8$, df =1)
Low risk – CIN II & III vs CIN I $p>0.10$ ($\chi^2=2.5$, df =1)

5.1.c. Association of HPV with different sociodemographic factors

Association of education, socioeconomic condition, age of first marriage and age of first delivery were analyzed considering all three groups of women together.

Relationship between education and HRHPV

Fig 5.3 revealed that women without formal education and with lower rate of secondary education had significantly higher HRHPV prevalence (p= 0.000).

Relationship between socioeconomic condition and HRHPV

Bivariate analysis did not show significant relationship (p=0.94) between socioeconomic condition and the prevalence of HRHPV (Table 5. 4).

Table 5. 4 : Relationship between Socioeconomic Condition and HRHPV (N=311)

Monthly Income (in taka)	HR HPV +ve group N=163	HR HPV -ve group N=148
Poor < Taka3000	104 (63.8)	95 (64.2)
Lower Middle, Higher Middle, Rich > 3000	59 (36.2)	53 (35.8)

$p = 0.94$ (χ^2=005, df =1)

Relationship between age of first marriage and HRHPV

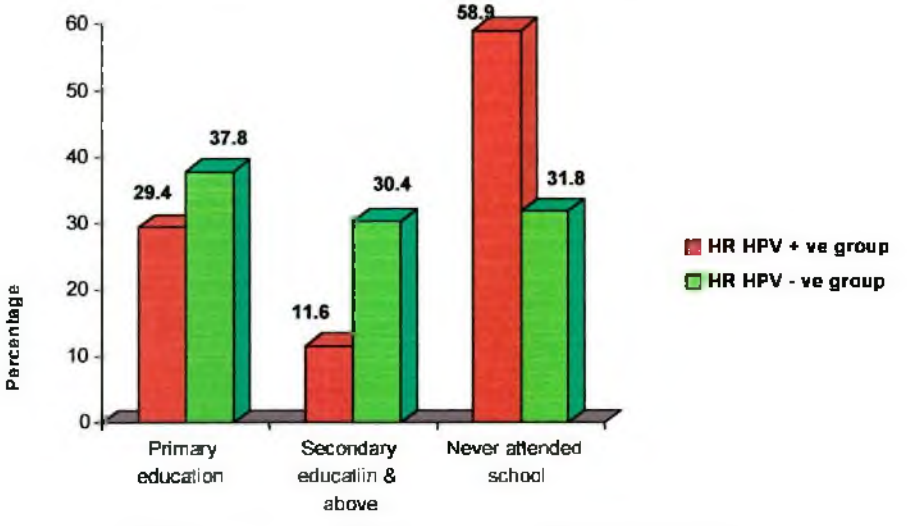
Considering all three groups of women together, the prevalence of HRHPV was significantly higher (p=0.000) among the woman who were married in earlier years of their life (Fig 5.4).

Relationship between age of first delivery and HRHPV

Considering all three groups of women together, the prevalence of HRHPV was significantly higher (p=0.007) among the woman who had their 1st delivery between 12-15 years of age (Table 5.5). Also women who were married after 20 years of age had significantly lower prevalence of HRHPV.

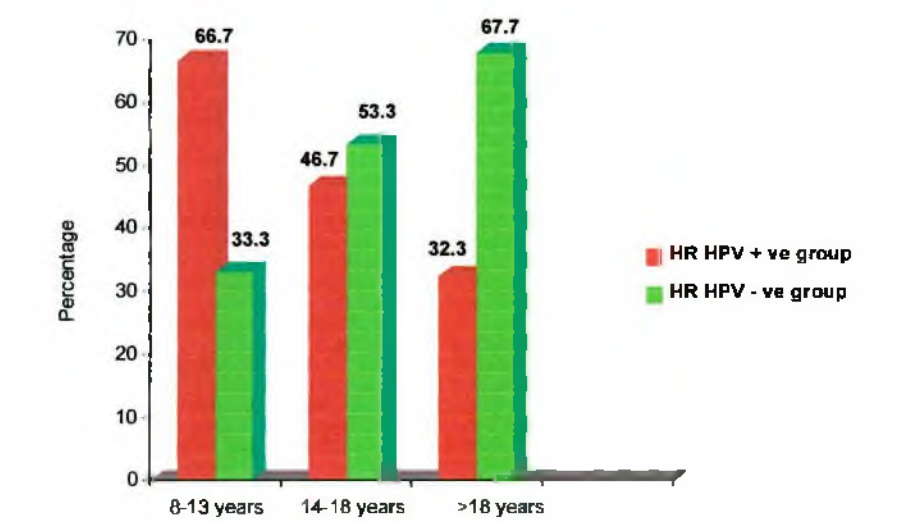
Table 5.5 : Relationship between Age of First Delivery and HRHPV (N=311)

Age at 1st pregnancy N=310	HR HPV +ve group N=162	HR HPV -ve group N=148	P value
12 -15	61 (37.4)	34 (23.0)	0.007
16-20	83 (50.9)	78 (52.7)	0.821
>20	18 (11.0)	36 (23.3)	0.002



HRHPV + ve group vs HRHPV -ve group p = 0.000 (χ^2= 29.54, df= 4)

Fig 5.3 : Relationship between education and HRHPV showing lower rate of secondary education and no education increases HRHPV prevalence (N=311)



HRHPV + ve group vs HRHPV -ve group p = 0.000 (χ^2= 16.27, df= 2)

Fig 5.4 : Relationship between age of first marriage and HRHPV showing higher prevalence of HRHPV among the group of women who were married early (N=311)

5.2 Association of other STAs with CIN and ICC

5.2.a. Influence of HSV II on CIN and ICC

In the present study HSV II infection did not carry increased risk for developing CIN or ICC. Seropositivity of HSV II antibody was almost simillar among women of all three groups (Fig 5.5).

5.2.b. Influence of *T. Pallidum* on CIN and ICC

Among the 120 ICC cases 16 (13.3%) were positive for TPHA. Five (7.1%) of 70 CIN cases were also positive for TPHA. None of the women of control group were seropositive for *T. Pallidum* and data analysis was not possible for risk assessment.

5.2.c. Influence of *N. gonorrhoea* Infection on CIN

Gonococcal DNA was tested only for the cervical samples of CIN and control cases (Fig 5.6). Among the 70 CIN cases, eight (6.6%) were positive for gonococcal DNA and this influence was statistically significant ($p<0.03$).

The independent influence of HRHPV and LRHPV and other genital tract infection on the development of CIN and ICC was assessed by logistic regression analysis (Table 5.6 and table 5.7). It was observed that the influence of HRHPV for development of CIN (OR=27.1, 95% CI 9.6-77.0) and ICC (OR=893.7, 95% CI 211.1-3784.1) remained as strong and independant influencing factor.

The influence of LRHPV for development of CIN (OR= 6.49, 95% CI 1.10-38.24) still remained as an influencing factor but it did not show any independent influence on development of ICC (OR= 1.8, 95% CI 0.3- 11.2). *N. gonorrhoea* though showed influence on development of CIN by bivariate analysis, during regression analysis this effect was nullified by the influence of HPV.

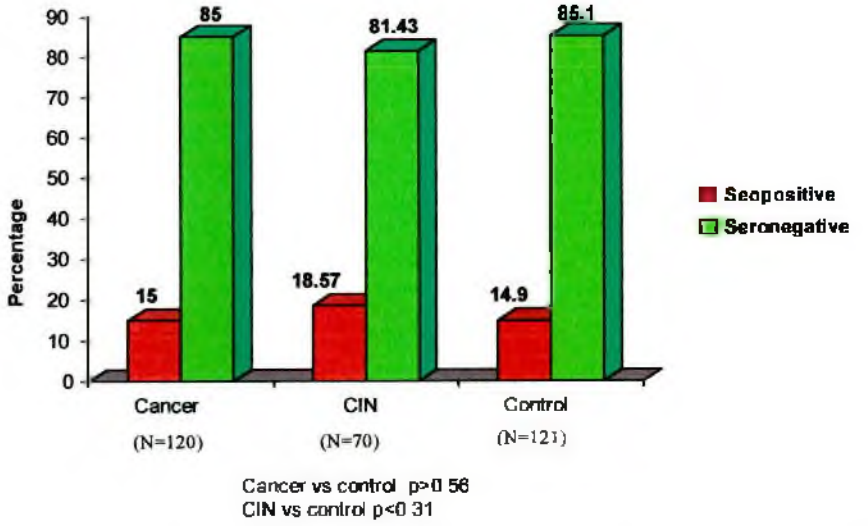


Fig 5.5 : Distribution of HSV II IgG antibody among the groupe showing no association of HSV II with CIN and ICC

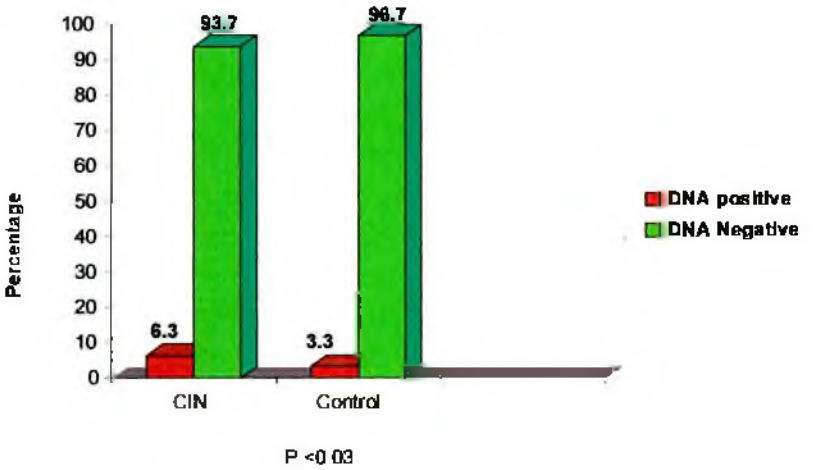


Fig 5.6 : *N. gonorrhoea* DNA prevalence in CIN and control showing association of gonococcal infection with CIN (bivariate analysis)

Table 5.6: Influence of STAs on Likelihood of Having CIN

Sexualy transmited agents	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
HRHPV					
Absent	1		1		
Present	5.9	0.000	27.1	9.6- 77.0	0.000
LRHPV					
Absent	1		1		
Present	17.6	0.000	6.5	1.1- 38.2	0.039
N. gonorrhoea					
Absent	1		1		
Present	3.8	0.036	2.9	0.6- 13.2	0.196
HSV II					
Seronegative	1		1		
Seropositive	1.0	0.505	1.7	0.6- 4.5	0.311

Table 5.7: Influence of STAs on Likelihood of Having ICC

Sexualy transmited agents	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
HRHPV					
Absent	1		1		
Present	0.04	0.000	893.7	211.1-3784.0	0.000
LRHPV					
Absent	1		1		
Present	0.14	0.01	1.8	0.3-11.2	0.56
HSV II					
Seronegative	1		1		
Seropositive	1.0	0.98	0.8	0.24-3.1	0.83

5.3 Influence of socioeconomic variables on CIN and ICC

The influence of socio-economic factors on development of cervical cancer was initially assessed by bivariate analysis. The independent influence of a particular parameter on development of CIN and ICC was assessed by regression analysis.

5.3.a. Influence of education of women

Better educational status have positive influence on occupation, increases age of marriage and helps in better attendance to health care services and all these factors may influence the process of carcinogenesis. Considerable number of women (68.3%) in the ICC group never attended school and only a few (5%) of them attended secondary education or higher. On the other hand 30.6% of the control group received secondary education or more (Table 5.8). These findings revealed significant risk of developing ICC among women of low level of education (p=0.000).

Women in the CIN group had a better educational status and 30% of them attended secondary education or more. No association was observed between CIN and low level of education in this study population (p>0.14).

Table 5.8: Education of Women

Levels of education	Cancer (N=120)	CIN (N=70)	Control (N=121)
Primary education	32 (26.7)	32 (45.7)	40 (33.1)
Secondary education & above	6 (5.0)	21 (30.0)	37 (30.6)
Never attended school	82 (68.3)	17 (24.3)	44 (36.4)

Cancer vs control - p=0.000 (χ^2=34.7, df=2)
CIN vs control - p=0.14 (χ^2=3.9, df=2)

Level of education influenced the age of marriage. During analysis women who never attended school and women who were educated up to primary level were grouped together and this group showed significantly higher rate of marriage (p=0.000) before 18 years of age than the women who attended secondary education (Table 5.9 and Table 5.10).

Table 5.9: Relation of Education of Women with Age of Marriage among ICC (N=241)

Levels of education	Age of marriage before 18 years	Age of marriage after 18 years
Never attended school, Primary education	189(95.5%)	9(4.5%)
Secondary education & above	28(65.1%)	15(34.9%)

p=0.000 (χ^2=36.26, df=1)

Table 5.10: Relation of Education of Women with Age of Marriage among CIN (N=191)

Level of education	Age of marriage before 18 years	Age of marriage after 18 years
Never attended school, Primary education	125(94.0%)	8(6.0%)
Secondary education & above	39(67.2%)	19(32.8%)

p=0.000 (χ^2=23.79, df=1)

5.3.b. Influence of occupation of the women

Majority of the women of all three groups were housewife, which reflected a poor social and occupational status of the women in this study group (Table 5.11). The results indicated that there was no association between women's occupation and development of the disease.

Table 5.11: Occupation of Women

Occupation of women	Cancer (N=120)	CIN (N=70)	Control (N=121)
Housewife	107 (89.2)	64 (91.4)	108(89.3)
Service holder, teacher, Labourer	13 (10.8)	6 (8.6)	13(10.8)

Cancer vs control - p= 0.982 (χ^2=0.001, df=1),
CIN vs control - p= 0.469(χ^2=1.5, df=1)

5.3.c. Influence of occupation of women's husband

Service holders, farmers, businessmans and teachers were grouped together during categorization of occupations of women's husband assuming that they enjoy a comparatively stable family and social life. Driver, conductors, police, BDR, mason, labourers were placed in a separate group. Among all these categories of occupation, none of them showed significant association (p=0.78) with cervical cancer (Table 5.12).

Table 5.12: Occupation of Women's Husband

Occupation of women's husband	Cancer (N=120)	CIN (N=70)	Control (N=121)
Service holder, teacher, Businessman, Farmer	87(72.5)	53 (75.7)	94(77.7)
Driver/ Conductor, Police/BDR, Labourer	33 (27.5)	17(24.3)	27 (22.3)

Cancer vs control - p= 0.78 (χ^2=3.1, df=1)
CIN vs control - p= 0.88 (χ^2=0.7,df=1)

5.3.d. Influence of income

There was no significant difference of economic status among the women (Table 5.13) of ICC and control group (p=0.525). It was interesting to observe that the women of CIN had comparatively better economic condition than the control group (p=0.003).

Table 5. 13: Different Income Groups in the Study Population

Monthly Income(in taka)	Cancer (N=120)	CIN (N=70)	Control (N=121)
Poor < Taka 3000	43 (35.8)	17 (24.3)	52 (43.0)
Lower Middle 3000-8000, Higher Middle 8000-12000, Rich> 12000	77 (64.2)	53 (75.7)	69 (57.0)

Cancer vs control - p=. 0.13 (χ^2=1.5, df=1)
CIN vs control - p=0. 01 (χ^2=6.7, df=1)

Multivariate regression analysis was done to assess the influence of individual socioeconomic variables on development of ICC and CIN (Table 5.14 and 5.15).

Lower education group were 3.32 times (95% CI 1.1 -10.1) more likely to develop ICC compared to better education group. Lower income group was 3.3 times (95% CI 1.2 -

9.2) more likely to develop CIN and 6.9 times (95% CI 2.1- 23.2) more likely to develop ICC compared to higher income group.

Regression analysis showed that though lower education and poor socioeconomic condition have significant influence on development of cervical cancer, it could not nullify the independent influence of HRHPV on development of CIN (OR=43.8, 95% CI 13.4-142.6) and ICC (OR=893.7, 95% CI 221.1-3784.0).

Table 5.14: Influence of Socioeconomic Variables on Likelihood of Having CIN

Socioeconomic factors	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Education of patient					
Educated group	1		1		
Non educated group	0.8	0.33	0.5	0.2-1.3	0.131
Monthly Income					
Middle class and Rich group	1		1		
Poor group	2.4	0.01	3.3	1.2-9.2	0.020
HRHPV					
No HRHPV	1		1		
HRHPV	5.9	0.000	43.8	13.4-142.6	0.000
LRHPV					
No LRHPV	1		1		
LRHPV	17.6	0.000	6.1	0.9-39.8	0.06

Table 5.15: Influence of Socioeconomic Variables on Likelihood of Having ICC

Socioeconomic factors	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Education of patient					
Educated group	1		1		
Non educated group	3.0	0.000	3.3	1.1– 10.1	0.03
Monthly Income					
Middle class and Rich group	1		1		
Poor group	1.3	0.26	6.9	2.1-23.2	0.001
HRHPV					
No HRHPV	1		1		
HRHPV	25.9	.000	893.7	211.1- 3784.0	0.000
LRHPV					
No LRHPV	1		1		
LRHPV	7.2	0.01	1.8	0.3-11.9	0.56

5.4 Influence of sexual behaviour on CIN and ICC

5.4.a. Age of menarche and first marriage

The mean age (Mean±SD) of menarche was 12.5±1.3, 12.6±0.2, and 12.8±1.2 years in the ICC, CIN and control group respectively. The differences among the three groups were not significant (CIN vs control- $p>0.10$, Cancer vs control- $p>0.05$).

The mean age (Mean±SD) at 1st marriage was 13.5±2.5, 15.6±3.5, and 15.7±3.5 years in the ICC, CIN and control group respectively (Fig 5.7) and early marriage had significant association with ICC ($p <0.001$). Therefore in the ICC group, the women were married early and nearer to menarche. The mean age of marriage of CIN was higher than ICC group and it was not statistically different from the control group ($p>0.10$).

The mean age (Mean±SD) at 1st intercourse was 13.8±2.2, 15.7±3.2 and 15.8±3.3 years in the ICC, CIN and control group respectively. There was no significant difference between the age of marriage and age of intercourse among the women of all three groups (ICC- $p>0.10$, CIN - $p>0.10$, control - $p>0.10$). So in this study, age of 1st marriage and age of 1st intercourse were used as synonym during further description.

About 96% of the women of ICC group were married before the age of 18 years (Table 5.14). Women who were married after 18 years had lower incidence of ICC (4 vs 116). Women who were married before 18 years had significant risk of having ICC ($p= 0.000$).

Table 5.14: Age at First Marriage

Age at 1st Marriage	Cancer (N=120)	CIN (N=70)	Control (N=121)
1st Marriage by the Age of eighteen	116 (96.7)	63 (90.0)	101 (83.5)
1st Marriage after the Age of eighteen	04 (3.3)	7 (10.0)	20 (16.5)

Cancer vs control - $p=0.000$ ($\chi^2=11.7$, df=1), Yates correction done
CIN vs control - $p=0.151$ ($\chi^2=1.6$, df=1)

5.4.b. Influence of number of marriage of woman

The mean number of marriage of woman was almost similar among all three groups (Table 5.15) and there was no significant association of number of marriage of women (p=0.492) with cervical cancer.

Table 5.15: Number of Marriage of Women

No of marriage	Cancer (N=120)	CIN (N=70)	Control (N=121)
1	110 (91.7)	66 (94.3)	112 (92.6)
2 and more	10 (8.3)	4 (5.7)	9 (7.4)
Mean±SD	1.1±0.3	1.1±0.2	1.1± 0.3

Cancer vs control - p=0.492 (χ^2=0.1, df=1)
CIN vs control - p= 0.447 (χ^2=0.2, df=1)

5.4.c. Influence of number of husband's marriage

The number of marriage of husband was significantly higher among the ICC group (Table 5.16) than that of the control group (p=0.045). Apart from that husband's number of marriage was significantly lower (p=0.003) in the CIN group than that of the control group.

Table 5.16: Number of Husband's Marriage

No of Husband's Marriage	Cancer (N=120)	CIN (N=70)	Control (N=121)
1	85 (70.8)	67 (95.7)	98 (81.0)
2 and more	35 (29.1)	3 (4.3)	23 (19)
Mean±SD	1.4± 0.8	1.1± 0.2	1.2± 0.5)

Cancer vs control - p =0.045 (χ^2=3.4, df=1)
CIN vs control - p =0.003 (χ^2=8.2, df=1)

5.4.d. Influence of sexually active period

The mean duration of sexual activity (Mean±SD) was 28.2±10.5, 18.7±7.2, and 24.1± 8.9 years in the ICC, CIN and control group respectively (Table 5.17). The increased duration of sexual activity had significant association with ICC (p=0.000). The women of CIN group had significantly lower duration of sexual activity than the control group (p= 0.000) and this was probably related to their younger age.

Table 5.17: Influence of Years of Sexual Activity

Number of years sexually active	Cancer (N=120)	CIN (N=70)	Control (N=121)
Upto 10 years	4 (3.3)	12 (17.1)	7 (5.8)
11 –20 years	20 (16.7)	32 (45.7)	41(33.9)
21 –30 years	47 (39.2)	20 (28.6)	41(33.9)
> 30 years	49 (40.1)	6 (8.6)	32 (26.4)
Mean±SD	28.9±10.5	18.7±7.1)	24.1±8.9)

Cancer vs control - t=3.81, p=0.000
CIN vs control - t= - 4.3, p=0.000

5.4.e. Influence of husbands travelling status

Higher number of husbands of the women of ICC and CIN group used to travel for working at different districts of the country (Table 5.18) and stayed separate for a considerable duration of time (>5 years, continuous or intermittent) than the control group (ICC group, p=0.002; CIN group, p=0.003). This finding indicates a significant association of husband's staying away from home for a considerable duration with cervical cancer. Only five husband of the ICC group and eight of them of the control group were living abroad or travelling abroad frequently. Five of the women of the ICC group has spoken about sexual exposure of their husband in brothel or with different partners.

Table 5.18 : Husbands Travelling Status

Husbands travelling status	Cancer (N=120)	CIN (N=70)	Control (N=121)
Living together	69 (57.5)	39 (55.7)	92 (76)
Lived separately for > five years	51 (42.6)	31(44.3)	29(24.0)

Cancer vs control - p=0.002 (χ^2=9.3, df=1)
CIN vs control - p =0.003 ((χ^2=8.5, df=1)

By regression analysis, the effect of number of husbands marriage, number of years of sexual activity and travelling status of husband and both risk groups of HPV showed independent influence on development of CIN (Table 5.19). On the other hand the early age of first marriage had seven times (OR=7.0, 95% CI 1.2- 40.7) and HRHPV had very strong association (OR=697.2, 95% CI 172.2-2822.9) with developing ICC. The effect of number of husbands marriage, number of years of sexual activity and travelling status of

husband and influence of LRHPV in the development of ICC did not exist after regression (Table 5.20).

Table 5.19 : Influence of Sexual Variables on Likelihood of Having CIN

Sexual Variables	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Age of Marriage					
18 years and above	1		1		
<18 years	1.8	0.216	3.4	0.9-12.5	0.07
Number of marriage of husband					
One marriage	1		1		
Multiple marriage	0.2	0.009	0.2	0.1 - 0.7	0.01
Duration of sexual activity					
upto 20 years	1		1		
> 20 years	1.5	.003	0.3	0.1-0.7	0.004
Travelling status of husbands					
Living together	1		1		
Lived separately for >5 yrs	1.2	.004	2.5	1.1-5.8	0.03
HRHPV					
Absent	1		1		
Present	5.9	0.000	30.7	9.9-94.9	0.000
LRHPV					
Absent	1		1		
Present	17.6	0.000	13.6	1.5-121.0	0.02

Table 5.20 : Influence of Sexual Variables on Likelihood of Having ICC

Sexual Variables	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Age of Marriage					
18 years and above	1		1		
<18 years	3.1	0.000	7.0	1.2- 40.7	0.03
Number of marriage of husband					
One marriage	1		1		
Multiple marriage	1.8	0.07	1.6	0.4-6.4	0.50
Duration of sexual activity					
upto 20 years	1		1		
> 20 years	2.7	.0001	1.8	0.6-5.0	0.27
Travelling status of husbands					
Living together	1		1		
Lived separately for >5 yrs	1.2	.003	2.2	0.8-6.0	0.13
HRHPV					
Absent	1		1	172.2-	
Present	25.9	0.000	697.2	2822.9	0.000
LRHPV					
Absent	1		1		
Present	7.2	0.01	4.5	0.5-43.0	0.19

5.5 Influence of reproductive status on development of CIN and ICC

5.5.a. Influence of age at first delivery

The mean age (Mean±SD) at 1st child birth was 16.5±2.9, 17.9±3.3 and 18.3±3.6 years in the ICC, CIN and control group respectively (Fig 5.8). Significantly higher number of women (p=.002) of the ICC had their 1st delivery before 15 years of age and among the control group significantly higher number of women (p=.003) had their 1st delivery after 20 years of age (Table 5.21). Also in the CIN group about 88% were married by 20 years of age and significantly lower number of women were married after 20 years than the control group.

Table 5.21 : Age at First Delivery

Age at 1st Delivery	Cancer (N=120)	Cancer vs control P value	CIN (N=70)	CIN vs control P value	Control (N=121)
12-15	51 (42.5)	.002	16 (22.9)	.96	28 (23.1)
16-20	55 (45.8)	.52	45 (64.3)	.06	61 (50.4)
> 20	13 (10.8)	.003	9 (12.9)	.03	32 (26.4)
No pregnancy	01 (0.8)	-	-	-	-
Mean±SD	16.5±2.9		17.9±3.3		18.3±3.7

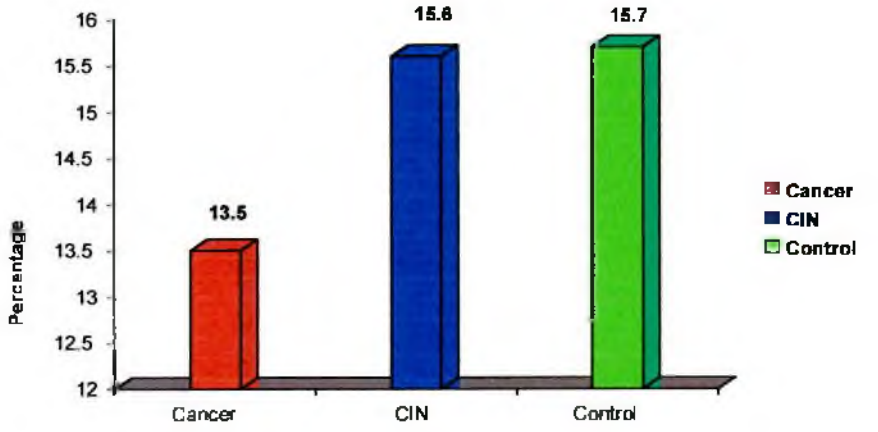
5.5.b. Influence of parity

The mean (Mean±SD) parity in the ICC group was 5.7±2.8. Table 5.22 shows that high parity had significant association with cervical cancer (p=0.03). Women of CIN group had significantly lower parity than the control group which seems related to their younger age.

Table 5.22 : Parity

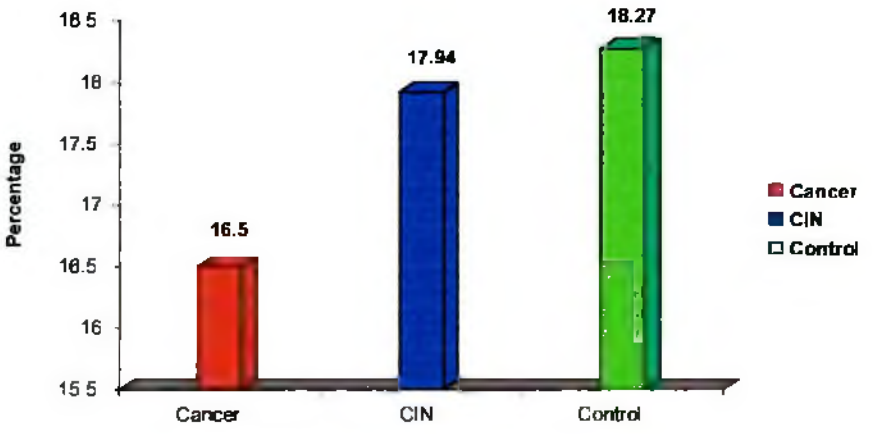
Parity	Cancer (N=120)	CIN (N=70)	Control (N=121)
0-2	11 (9.2)	26 (37.1)	25 (20.7)
3-6	69 (57.5)	40 (57.1)	68 (56.2)
7-9	40 (33.3)	4 (5.7)	28(23.2)
Mean±SD	5.7±2.8	3.4±2.0	4.6±2.5

Cancer group p =0.036 (χ^2=8.5, df=3)
CIN group p=0.002 (χ^2=12.5, df=2)



Cancer vs control p <0.001 ($\chi 2$ = 16.0, df = 2)
CIN vs control p = 0.069 ($\chi 2$ = 5.3, df = 2)

Fig 5.7 : Mean age of 1st marriage showing significantly lower mean age among ICC group



Cancer vs control p<0.001 (χ 2= 16.0, df= 2)
CIN vs control p= 0.07 (χ2= 5.3, df= 2)

Fig 5.8 : Mean age at first delivery showing significantly lower age of delivery among the ICC group

5.5.c. Influence of number of total pregnancy

Data on number of total number of pregnancies were presented in table 5.23. The mean number of pregnancies (Mean±SD) in the ICC for those who had ever been pregnant was 6.5±3.0 and was higher than the control group and the association had borderline significance only. One patient in the ICC group did not experience any pregnancy and five had only one pregnancy. Women of CIN group had significantly lower number of total pregnancy than the control group which may be related to their younger age.

Table 5.23 : Number of Total Pregnancy

Number of total pregnancy	Cancer (N=120)	CIN (N=70)	Control (N=121)
0-2	7 (5.8)	13 (18.6)	13 (10.7)
3-6	55 (45.8)	46 (65.7)	67 (55.4)
7-9	58 (48.3)	11 (15.7)	41(33.8)
Mean±SD	6.5±3.0	4.7±2.5	5.6±2.7

Cancer group p=0.065 (χ^2=7.238, df=3)
CIN group p=0.01 (χ^2=8.17, df=2)

When logistic regression analysis was applied to all these reproductive factors along with both risk groups of HPV to assess the independent influence of these factors on CIN, none of the mentioned reproductive factors showed independent influence and only the influence of HRHPV (OR=28.4, 95% CI 9.8-82.2) remained significant (Table 5.24).

On the other hand, when the independent influence of reproductive factors on development of ICC was assessed, early age at 1st delivery (OR=2.6, 95% CI 1.0-6.8) and parity eight and above (OR=17.7, 95% CI 1.4-228.1) were seen to have independent risk of developing ICC (Table 5.25). When the analysis considered the effect of parity less than eight, it did not show any significant association with cervical cancer. In contrary to that the borderline association of number of total pregnancy with cervical cancer was nullified. HRHPV also showed highly significant association (OR=763.3, 95% CI 182.4-3193.3) with ICC.

Table 5.24 : Influence of Reproductive Status on Likelihood of Having CIN

Reproductive Status	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Parity					
upto eight	1		1		
> eight	2.3	0.001	2.9	0.2-35.1	0.392
No of pregnancy					
upto eight	1		1		
> eight	2.0	0.007	0.2	.03- 1.2	0.08
Age of Ist delivery					
>18 years	1		1		
upto 18 years	1.4	0.186	1.16	0.5-2.5	0.700
HRHPV					
Absent	1		1		
Present	5.9	0.000	28.4	9.8- 82.2	0.000
LRHPV					
Absent	1		1		
Present	17.6	0.000	5.4	0.9- 31.9	0.060

Table 5.25 : Influence of Reproductive Status on Likelihood of Having ICC

Reproductive Status	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Parity					
upto eight	1		1		
> eight	1.4	0.17	17.7	1.4- 228.1	.03
No of pregnancy					
upto eight	1		1		
> eight	1.2	0.50	0.3	0.03- 1.8	0.16
Age of Ist delivery					
>18 years	1		1		
upto 18 years	2.3	0.003	2.6	1.0- 6.8	0.05
HRHPV					
Absent	1		1		
Present	25.9	0.000	763.3	182.4- 3193.3	0.000
LRHPV					
Absent	1		1		
Present	7.2	0.01	1.5	0.2- 10.3	0.67

5.6 Influence of contraceptive practice on CIN and ICC

The influence of different contraceptive practices were difficult to analyze due to infrequent use of some of the methods. There was also difficulty to observe the influence of contraceptive methods on CIN as age of the women of CIN group was not matched with that of the control group.

In this study oral contraceptive pill was the most widely used contraceptive method (48.1%) and injectable contraceptives were used by 12.2% of them. None of the husband of all three groups had vasectomy as a method of contraception. However, 11.2% of all the women had tubectomy (Table 5.26).

Table 5.26 : Contraceptive Practices

Methods of contraception	Cancer (N=120)	CIN (N=70)	Control (N=121)	Average
Barrier method	3 (2.5)	13 (18.6)	20 (16.5)	12 (12.5)
OCP	48 (39.6)	46 (65.7)	47 (38.9)	47 (48.1)
Inj	18 (14.8)	8 (11.4)	13 (10.7)	13 (12.2)
IUD	5 (4.1)	7 (10)	8 (6.6)	6.7 (6.9)
Tubectomy	11 (9.1)	8 (11.4)	16 (13.2)	11.7 (11.2)
No contraception	56 (46.3)	14 (20.0)	-	-

Among the different contraceptive methods, bivariate analysis showed that OCP was associated with high incidence of CIN and barrier method was associated with low incidence of ICC. On the other hand, multivariate analysis showed that none of the contraceptive methods influenced independently in the process of cervical carcinogenesis (Table 5.27 and 5.28). However, the very strong individual carcinogenic influence of HRHPV (OR=906.4, 95% CI 212.6-3864.7) was not nullified by influence of different contraceptive methods.

Table 5.27 : Influence of Contraceptive Practice on Likelihood of Having CIN

Contraceptive methods	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Barrier					
Barrier not used	1		1		
Barrier used	1.0	0.719	2.2	0.8-5.5	0.120
OCP					
OCP not used	1		1		
OCP used	1.2	0.000	1.8	0.8-4.1	0.144
Inj					
Injection not used	1		1		
Injection used	1.1	0.884	0.8	0.2-2.7	0.711
IUD					
IUD not used	1		1		
IUD used	1.1	0.40	0.6	0.1-2.7	0.477
Tubectomy					
Not done	1		1		
Tubectomy done	2.0	0.719	1.4	0.4-4.5	0.584
HRHPV					
Absent	1		1		
Present	0.0	0.000	27.9	9.3-83.2	0.000
LRHPV					
Absent	1		1		
Present	0.1	0.01	6.2	1.0-36.6	0.045

Table 5.28 : Influence of Contraceptive Practice on Likelihood of Having ICC

Contraceptive methods	Unadjusted		Adjusted		
	Odds Ratio	P value	Odds Ratio	95% CI	P value
Barrier					
Barrier not used	1	0.001	0.4	0.2-2.2	0.27
Barrier used	0.8				
OCP					
OCP not used	1				
OCP used	1.0	0.96	0.4	0.1-1.1	0.06
Inj					
Injection not used	1				
Injection used	1.1	0.42	1.2	0.3-5.3	0.78
IUD					
IUD not used	1				
IUD used	0.9	0.40	0.2	0.1-1.2	0.07
Tubectomy					
Not done	1				
Tubectomy done	0.9	0.32	0.6	0.1-3.0	0.58
HRHPV					
Absent	1				
Present	0.1	0.000	906.3	212.6-3864.7	0.000
LRHPV					
Absent	1				
Present	0.2	0.01	1.5	0.2-10.2	0.70

Independent influence of HRHPV and LRHPV on development of CIN was observed along with socioeconomic factors (HRHPV- OR= 43.8, 95% CI 13.4-142.6; LRHPV- OR=6.1, 95% CI 0.9-39.8), sexual behaviour (HRHPV- OR=30.7, 95% CI 9.9-94.9; LRHPV- OR=13.6, 95% CI 1.5-121.0), reproductive status (HRHPV- OR=28.4, 95% CI 9.8-82.2; LRHPV- OR=5.4, 95% CI 0.9- 31.9), contraceptive factors (HRHPV- OR= 27.9, 95% CI 9.3-83.2; LRHPV- OR=6.2, 95% CI 1.1-36.6). All the results show very strong association of HRHPV with CIN. Contrarily LRHPV did show some association with CIN when sexual and contraceptive factors were considered.

Independent influence of HRHPV and LRHPV on development of ICC was observed along with socioeconomic factors (HRHPV- OR=893.7, 95% CI 211.1-3784.0); LRHPV- OR=1.8, 95% CI 0.3-11.9), sexual behaviour (HRHPV- OR=697.2, 95% CI 172.2-2822.9; LRHPV- OR= 4.5, 95% CI .5- 43.0), reproductive status (HRHPV- OR= 763.3, 95% CI 182.4-3193.3; LRHPV- OR=1.5, 95% CI 0.22-10.3), contraceptive factors (HRHPV- OR=906.3, 95% CI 212.6-3864.6; LRHPV- OR=1.5, 95% CI 0.21-10.2). All the results showed very strong association of HRHPV with development of ICC. Contrarily LRHPV did not show any effect on development of cervical cancer.

CHAPTER 6

DISCUSSION

CHAPTER 6

DISCUSSION

Genital HPV is established as a major factor for cervical carcinogenesis. Nevertheless, detailed information about the prevalence of this infection and the association of possible risk factors has not been adequately studied in many population. The present research work has been undertaken to study the prevalence of both HRHPV and LRHPV among women with ICC, CIN and normal cervix. The study also tried to find out the association of different risk factors for development of ICC and CIN in Bangladeshi population.

The women of CIN group were recruited from gynaecological OPD of BSMMU. Though the women of ICC and control group were recruited after matching of age, the same was not possible during recruitment of the women of CIN group. The mean age of CIN was significantly lower than the control group. This made their parity, number of marriage of husband, years of sexual activity lower and this drawback produced difficulties in interpretation of results. Multivariate regression analysis helped to overcome these drawback to some extent.

It was revealed that nearly half of the patients of ICC group were living in urban areas of the country where primary health care and facilities for routine gynaecological checkup were available along with some modalities of treatment of CIN and ICC. Utilization of these facilities could prevent or delay the mortality and morbidity of these women. Unfortunately, they did not avail the accessible benefits because of unawareness of the disease process, it's consequences, availability of gynaecological check-up and it's benefit. Development of awareness regarding natural history of cervical cancer and importance of cervical examination is of paramount importance for early diagnosis of preinvasive and invasive cervical carcinoma and it's prevention and management.

Age Distribution

This study found that about one fifth of ICC patients were upto 40 years of age. Thereafter, incidence increased with increase of age and 80% of them presented during 4^{th} - 6^{th} decade. This age distribution reflected the late presentation of the cancer patients to the physician in Bangladesh. Kurohara et al (1970) found a similar age distribution among the ICC patients in United States of America (USA) before 1960s when screening of cervical cancer was almost non-existent in that country. They collected data from medical records of 2363 women of ICC during 1920-1953 in New York. These retrospective data showed that about 20% of the ICC presented before 40 and 80% after 40 years of age (Kurohara et al 1970). The International Agency for Research on Cancer (IARC) working group described almost a similar age distribution and mentioned that ICC in women under 25 years of age was rare (IARC working group 1986). All these findings indicated that during development of a screening programme, priority to women of 35-40 years at the initial stage of the programme may help in identifying more cases of the ICC and CIN within a shorter period of time.

The mean age (Mean±SD) of the patients in this study with ICC, CIN and control was 47.2±11.2, 34.9±8.2 and 45.5±10.7 years respectively. Brinton et al (1989) revealed a mean age of 46.5 year in a case control study in Latin America and review of the cancer registry of Rajsthan and Maharastra of India also showed a similar finding (Kulkarni et al 1996, Sharma et al 1994). The similarity of the mean age of ICC of the present study with those studies is probably due to the lack of screening programme at the national level in the mentioned countries. On the other hand, the mean age of patients of ICC in a hospital based study in Freiburg of Germany was 56.8 ±15.5 years (Ikenberg et al 1994)]. This higher mean age was probably related to existence of screening programme within that population. Therefore, the mean age of ICC is comparatively high in countries where cervical cancer screening programme is existing.

6.1 Association of HPV infection with CIN, ICC

Evaluation of diversity of HPV in various grades of cervical lesion is important for understanding the characteristics of HPV infection in the pathogenesis of cervical neoplasia. Genital tract HPV infection is extensively present throughout the world. However, detailed information about the prevalence of this infection and its association with other risk factors were inadequately studied in the developing countries. The epidemiological study of HPV has been limited by less availability of HPV detection techniques. The available investigations for HPV detection are expensive and not readily available in majority of the developing countries and not always feasible to use in large population.

This study showed that HRHPV was the strongest independent determinant factor for developing ICC and CIN in Bangladeshi women. HPV DNA was detected using HC II technology which differentiated between HRHPV (16/18/31/33/35/39/45/51/52/58/59/ 68) and LRHPV (6/11/42/43/44) types. This technique is available only for last few years and hence prevalence study using this method is only recently done. The quality control of the study was strictly followed by running three negative control and three positive calibrator with each test run. The prevalence of HRHPV among the ICC patients is quite high in this study (96.7%) than many of the mentioned study done using different methods in different populations. High sensitivity (81%) of HC II (Womack 2000), the technique used in this study may be related for this high prevalence.

The results of this study have confirmed that HRHPV infection is present in the vast majority of cervical cancer of this population and plays central role in the development of CIN and ICC. The HRHPV was prevalent among 96.7% of the ICC. The independent influence of HRHPV, LRHPV and other genital tract infection on the development of ICC was separately assessed and the influence of HRHPV always remained as a very strong influencing factor. Regression analysis was performed after inclusion of HRHPV and LRHPV DNA report in different models separately (socioeconomic, sexual, reproductive and contraceptive factors). All results revealed strong independent association of HRHPV with CIN and ICC.

A cross-sectional study in California demonstrated HPV infection as the key risk factor for cervical carcinogenesis and mentioned about the sexual route of HPV transmission (Ley et al 1991). Munoz et al (1992) in a population based case-control study measured HPV DNA seperately by three different hybridization techniques (ViraPap, SBH and PCR) in Spain and Columbia. They proved very strong and causal association between HPV 16, 18, 31, 33, 35 and ICC. Another extensive epidemiological review concluded that over 90% of all ICC could be attributed to certain oncogenic HPV genotypes (Munoz and Bosch1996).

The obtained prevalence of HRHPV in Bangladeshi women with cervical cancer is significantly high and represent the prevalence of a developing country. This study was a cross sectional study on prevalence of HPV comparing ICC, CIN and normal cervix. Data using similar technique was inadequate in this country for comparison. It was thought to be worthwhile to compare the prevalence of HPV of this study with those of other studies done in developed and developing countries. During comparison of HPV prevalence of different populations, the discussion has been categorized into three groups: as ICC (Table 6.1), CIN (Table 6.2) and control (Table 6.3) group. Different studies assessed this infection using different methodology and laboratory techniques which made interpretation and comparison difficult.

Table 6.1 described the prevalence of HPV among ICCs of different populations. Filter *in situ* hybridization (FISH) and SBH were used widely for detection of HPV in 1970's and 1980's. The detection rate of HPV by FISH among the ICC samples ranged between 67-72% (Reeves et al 1987, Schneider et al 1987). The SBH showed almost a similar detection rate as FISH and HPV prevalence varied between 62-73% in United Kingdom, France, Algeria and Germany (Ikenberg et al 1994, Riou et al 1990, Meanwell et al 1987). Chatterjee and Basu (1995) revealed a low prevalence of HPV DNAs (50%) in the ICC biopsy specimens in Calcutta, India. The use of commercial Vira Type kits may be a reason of comparatively lower prevalence of HPV DNA in that study.

By nineties PCR came into practice by different countries and this new method improved the detection rate of HPV. Bosch et al (1995) conducted a large multinational survey by the International Biological Study on Cervical Cancer (IBSCC) and collected sample

from 22 countries of Africa, Central and South America, Southeast Asia, North America and Europe (866 samples). They reported a worldwide HPV prevalence of 93% in ICC specimens using PCR and revealed that there was no significant variation in HPV prevalence among ICC specimen of different countries. In that study about 26 different type specific probes were used and HPV 16 was present in 50% of the specimen, HPV 18 in 14%, HPV 45 in 8% and HPV 31 in 5%.

However, Walboomers et al (1999) showed a higher prevalence of HPV in cancer specimens by continuing the study done by Bosch et al in 1995. They worked on 58 formalin fixed, paraffin-embedded tissue specimens of the 66 HPV-negative cases of IBSCC study. By performing HPV E7 type-specific PCRs, majority of the negative cases of ICC showed presence of HPV DNA and when this result was combined with data of IBSCC data of Bosch et al, the worldwide HPV prevalence in ICC was revealed as 99.7%. Their PCR results indicated that many of the samples which were false negative in the study of Bosch et al (1995) came as positive for HPV DNA.

In this study, the HRHPV was prevalent among 96.7% of the ICC and four cases were HPV negative. By performing HPV E7 type-specific PCRs, this four negative cases could be tested for presence of HPV DNA. But this procedure was not included in the methodology of this study and was not possible to perform.

This study noticed the association of HPV with all grades of CIN (Table 5.2). The intensity of association increased with higher grade of the lesion, and the association was most noticeable with the HRHPV types. HRHPV were more significantly associated with CIN II and III than CIN I group (Table 5.3). This increased prevalence of HRHPV in high grade lesions indicated the role of HRHPV in development of CIN and progression of the condition to high grades of lesions and ultimately to ICC. A large community based study in rural Taiwan also showed an upward trend of HPV DNA (all types) prevalence (PCR) from CIN I (54%) to CIN II / III (92%) (Liaw et al 1995). Ferrera et al (1999) observed a statistically significant association of HRHPV with CIN II, CIN III and invasive cancer, showing an upward trend to more severe lesions.

Table 6.1 Prevalence of HPV in ICC of Different Countries:

Author	Type of study	Country/ Population	Method	HPV type	HPV prevalence
Schneider et al 1987	Cross sectional	Germany	FISH	16 and 18	72%
Meanwell et al 1987	Case -control	West Midlands, UK	SBH	6, 11, 16, 18, 31, 33, 35	62 - 73%
Reeves et al 1989	Case -control	Panama, Costa Rica and Colombia 1986-87	FISH	6, 11, 16 and 18	62%
Riou et al 1990	Cross sectional	France and Algeria	PCR and SBH	16, 18, 33, 35 and uncharacterised	84%
Ikenberg et al 1994	Cross sectional	Germany	PCR and SBH	16, 18, 31, 33, 35	73%
Chatterjee et al 1995	Cross-sectional	Calcutta, India	Vira Pap and Vira Type kits	6, 11, 16, 18, 31, 33, 35	50%
Labropoulou et al 1996	Cross sectional	Greece	PCR and SBH	16, 18, 31, 33, 51	89% (PCR) 61% (SBH
Chen et al 1999	Hospital based, cross sectional	Australia 1989-94	PCR	16, 18, 31,33,45	91.9%
Herraro 2000	Population based, cohort	Costa Rica 1993 -1994	PCR	16, 18, 31, 33,35,39,45,51,52, 56, 58,59,68	88%

The independent association between HRHPV and CIN remained very strong even after adjustment with socio-demographic factors and other genital tract infection (HSV II, *N. gonorrhoea*). All these results therefore supported the role of HRHPV as a strong independent factor for developing and progression of the disease.

Table 6.2 revealed the prevalence of HPV among CIN of different countries. Majority of the research works related to HPV DNA prevalence of CIN in different study population has been carried out in 1990s and that was probably the reason behind using PCR by almost all of them. The HPV prevalence varied from 54% in CIN I to 96.5% when all grades of CIN were included. The variation of the result may also be related to variation of detection methods used, grade of CIN and difference of geographical areas. When studies included only CIN II and CIN III, HPV DNA (PCR) prevalence ranged between 80-96% (Tabrizi et al 1999, Kjellberg et al 1998, Kjaer et al 1996, Olsen et al 1995).

In this study both bivariate and multivariate analysis showed LRHPV had significant association with CIN. This occurred as a result of inclusion of a large number of CIN I (about 50%) which contained more LRHPV than HRHPV. Kjaer (1996) in Denmark also showed an independent association of LRHPV with CIN and the prevalence of LRHPV was 21.7% when he included all grades of CIN. However, LRHPV did not have any independent influence in developing ICC. Lorincz et al (1992) in a case control study also showed that LRHPV was present 20.2% in low grade lessions. All these findings indicated a role of LRHPV in developing CIN, but not in ICC. This information is particularly important in managing CIN by avoiding unnecessary intervention. Follow up is justified for LRHPV+ve and HRHPV-ve CIN patients. Hall et al (1996) also found a mixed HRHPV and LRHPV infections in 15% of CIN lesions. The association was more with CIN I and this indicate that low grade lesions are more related to LRHPV and this information points that intervention in the low grade lesion is unnecessary.

Table 6.2 Prevalence of HPV in CIN of Different Countries:

Author	Type of study	Country/ Population	Method	HPV type	HPV prevalence (%)
Das et al 1989	Cohort	Delhi,India 1976-88	ISH	16, 18	68% (CIN I, II, III)
Bosch et al 1993	Case-control	Spain and Colombia 1985-88	PCR	6, 11, 16, 18, 31, 33, 35	Spain -70.7% Colombia–63.2% (CIN III)
Schiffman et al 1993	Case-control	Portland, Ore 1989-90	PCR	6, 18, 31, 33, 35, 39, 45, 51, 52	76% (HSIL and LSIL)
Olsen et al 1995	Population based case-control	Norway 1991-92	PCR	6, 11, 16, 18, 31, 33	91% (CIN II &III)
Liaw et al 1995	Case-control	Taiwan 1991	PCR	6,11,16,18,26,31,33,35,39,40, 42, 45,51-59,68	92% (CIN II &III)) 54%(CIN I)
Hall et al 1996	Cross Sectional	USA, 1991 - 92	Hybrid Capture	6,11,42,43,44 16,18,31,33,35,45,51,52,56	HRHPV-93% LRHPV-23% (CIN I,II,III)
Kjaer et al 1996	Case-control	Denmark 1991-93	PCR	6, 11,16,18 and 33	71.2% (CIN I,II,III)
Kjelberg et al 1998	Population based case-control	Sweden, 1993-95	PCR	11,16,18 and 33	96% (HSIL)
Yishikawa et al 1999	Hospital based, case-control	Japan 1995-96	PCR	6,16,18,31,33,42,51,53,54,56,58,59, 61,66,68 and 70	76.6% (CIN I, CIN II and CIN III)
Tabrizi et al 1999	Cross sectional	Australia	PCR	6,11,16,18,31,33,35,39,45,51,52	89% (CIN II,III)

From the findings of this study, it become apparent that HPV infection is not so prevalent in the normal female population of Bangladesh. Our study showed that 5.8% of the control group harboured HPV DNA and 4.1% of them were HRHPV positive. Table 6.3 revealed the prevalence of HPV among control population of different countries. A study among women of major ethnic groups (Chinese, Indians and the Malays) in Singapore with normal Pap smear showed a HPV prevalence rate (FISH) of 5.9% (Chow et al 1990). No significant difference of HPV prevalence were found among these different ethnic groups of South East Asia and this prevalence was almost similar to the prevalence of the control of this study.

The control women of Norway, Denmark and Japan revealed almost similar (10 - 15%) HPV DNA (PCR) prevalence (Kjaer et al 1996, Olsen et al 1995, Nishikawa et al 1991, Yoshikawa et al 1991). In contrary to these results, Kjelberg et al (1998) showed a low prevalence of HPV among control population of sweden. The study in Sweden was a population based study and this may be related to comperatively lower prevalence of HPV in that population.

The study by Meanwell et al (1987) showed a comparatively higher (35%) HPV prevalence (SBH) in population of UK with normal cervices. This incidence seems quite high and this may be related to a high prevalence of STAs in the population studied. Ferrera et al (1999) showed a similar HPV DNA prevalence (PCR) among control women (39%) in a population-based, case-control study in Hondurus. The high prevalence of HPV among that control may be related to a high risk population and high-sensitivity of PCR sequensing approach used, which could detect more transient infections. Reeves et al in a multicentre case-control study in Panama, Costa Rica and Colombia (FISH) revealed an unexpectedly high prevalence of HPV (32%) among control women (Reeves et al 1989). However, the specimen was collected from hospitals of cities and not from the community. Also this high prevalence was probably related to characteristics of that particular population as poor socioeconomic condition, more pre-marital and extramarital relationships and high prevalence of other STAs and different social custom.

Table 6. 3 Prevalence of HPV in Control of Different Countries:

Author	Type of study	Country/ Population	Method	HPV type	HPV prevalence (%)
Meanwell et al 1987	Case-control	UK 1984-1985	SBH	6, 11, 16, 18, 31, 33, 35	35%
Reeves et al 1989	Multicentre Case-control	Panama, Costa Rica and Colombia 1986-87	FISH	6/11, 16 and 18	32%
Chow et al 1990	Cohort	Singapore 1989	FISH	11, 16, 18, 31, 33	5.9%
Nishikawa et al 1991	Cohort	Japan	PCR	16,18,33	5-10%
Bosch et al 1993	Case-control	Spain and Columbia 1985-88	PCR	High risk and Low risk	Spain 4.7% Colombia 10.5%
Schiffman et al 1993	Case-control	Portland, Ore 1989-90	PCR	6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-55, 57, 59	HRHPV (6.2%) LRHPV (11.5%)
Liaw et al 1995	Case-control	Taiwan 1991	PCR	6,11,16,18,26, 31, 33,35,39,40,42,45,51-59,68	9%
Olsen et al 1995	Population based case-control	Norway 1991-92	PCR	6, 16, 18, 31, 33	15%
Kjaer et al 1996	Case-control	Denmark 1991-93	PCR	16, 18, 31, 33	15.4%
Kjelberg et al 1998	Population based case-control	Sweden, 1993	PCR	11,16,18, 33	4%
Ferrera et al 1999	Hospital-based case-control	Honduras 1993-95	PCR	6,11,21,22,16,18,31,33,35, 45,52,53,55,56,58,59,62,66, 70	39%
Yoshikawa et al 1999	Hospital based, case-control	Japan 1995-96	PCR	6,16,18,31,33,42,51,53,54 ,56,58,59,61,66,68 and 70	14.6%

In a clinic based case control study in the University of New Mexico, among the control women 13.9% were HPV positive by Virapap and 42.12% by PCR. So the detection rate is quite high with PCR than Virapap (Becker et al 1994).

So published studies on the epidemiology of HPV infection showed considerable differences in prevalence among control population of different areas which may be related to the existence of different risk factors in different population in different geographical areas and variation of the results. The variation of the result is presumably due to existence of a variety of sampling methods for collection of specimen and the broad range of detection methods. The rate of HPV detection has been shown to vary, depending on which test is used (Herrington et al 1995).

The prevalence of HPV in majority of the Western and African countries among control population is higher than this study. Therefore the prevalence of HPV among the normal population of Bangladeshi women is comparatively low even after use of a sensitive method than the prevalence of Western and African countries. This may be related to the sociocultural background, less extramarital and premarital sexual experience, customs, family bonding etc present in this country.

Majority of the control group showed in table 6.3 had normal Pap smear only and control group was not selected on the basis of normal colposcope findings. On the other hand, during selection of women of control group in this study, women with normal Pap smear and colposcope findings were recruited only. Women with exessive vagival discharge were also not included in the control group. This probably created a difference and showed a lower HPV prevalence among the control women of this study.

It is observed that there can be wide variation of HPV prevalence among control population globally in different countries. Bosch et al showed that there was no difference of HPV prevalence among cervical cancer patients in different countries. But the prevalence of this infection varies distinctly between and within control population (Bosch et al 1995). The information of prevalence among the normal population is important in developing national screening programme.

6.2 Association of other STAs with CIN and ICC

Several epidemiological studies have linked HSV II infections to an increased incidence of ICC (Koffa et al 1995, Kessler 1977, Rawls et al 1970, Rawls et al 1969). Nehmias et al (1970) in a case control study demonstrated a gradually increasing seroprevalence of HSV II among women from cytologically diagnosed atypia to ICC for all ages. Studies indicated that both HSV II and HPV may act synergistically for developing cervical cancer, but the mechanism involved are different (Hildesheim et al 1991, Xiang-jin et al 1989). The studies also suggested that infection with both viruses is a significant risk but that infection with one or the other is not nearly as significant. Thus, HSV II may be a cofactor in some cases of cervical cancer. Case control studies in India showed association between cervical cancer and HSV II antibodies (Lakshmi et al 1993, Luthra et al 1987).

However study by Prakash et al (1985) did not show any evidence of interaction between HSV II and HPV 16 among the cases of cervical carcinoma. Several other studies also did not support the hypothesis that HSV II is an etiologic agent for cervical neoplasia (Dillner et al 1994, Sanjose et al 1994, Hakama et al 1993, Jha et al 1993, Lehtinen et al 1992, Lewis et al 1965). Kjaer et al (1990) in a population based case-control study in Greenland and Denmark assessed seroprevelance of HSV II antibodies. The study showed that CIN had no association (OR=1.1, 95% CI 0.5-2.2) with any elevation of HSV II risk.

The present study also could not identify any association between HSV II seroprevalence and CIN and ICC. Seropositivity of HSV II was almost simillar among women of all three groups.

The link between bacterial infections and carcinogeneis is not clear. Syphilis is a complex disease which is usually sexually transmitted and understanding syphilis in the pathogenesis of cervical cancer is worthwhile. Study on this particular field relating to cancer so far is inadequate. The causative organism, *T pallidum* can not be grown on conventional laboratory culture media or in the tissue culture. Infection is normally diagnosed by directing antibodies specific for *T. pallidum* in the patient's blood.

Though statistical analysis was not possible to compare the serorevalence of *T. pallidum* antibodies (13.3% among ICC and 7.1% among CIN) with the control as none of the women of control group was seropositive for *T. pallidum*, the association of this infection in development of ICC and CIN is an obvious finding. It is very difficult to obtain reliable information about syphilis from personal interview, therefore some weight can be attributed to the obtained serological results of this study. The infection and the malignancy may be co-variables with sexual promiscuity.

The patients in this study were not matched for sexual promiscuity. The present study revealed that number of syphilis cases occured more frequently in patients with CIN and ICC. Wynder et al (1954) also reported twice as many syphilitic patients among women with cervical cancer than the controls. The present study failed to compare the cases with the control as large sample size is needed for the control as it is less prevalent in the general population. So study with larger sample size of the control population is necessary to explore this area.

In this study, gonococcal DNA were tested in CIN and control cases and though bivariate analysis showed that presence of this infection had significant association with development of CIN, in multivariate regression analysis this effect was nullified by the strong influence of HPV. The findings of this study reflected that infections with *N. gonorrhoeae* may be a surrogate markers of HPV and it is difficult to identify whether this STA has separate etiological significance. In a population-based case-control study among Spanish women, an increase in risk of ICC was found among seropositive women of *N. gonorrhoeae* (De Sanjose et al 1994).

Another study concluded that malignant disease of the cervix proved at least four times common among the women with gonorrhea than the controls. It may be assumed that at least every fourth woman who had gonorrhea was a carrier of the carcinogenic agent (Herrero et al 1990). All these findings corroborate the view that cervical carcinoma is a sexually transmitted disease.

This study clearly established a strong independent influence of HRHPV infection in developing ICC and its precursors and came to a conclusion that this infection is the

primary cause of cervical cancer among Bangladeshi women. Factors like reproductive characteristics and genital infections can be interpreted as expressions of sexual behaviour. Despite the improving social status, increasing health consciousness and extensive mass-screening for cervical cancer, the above mentioned risk factors still play an important role. Women with history of HRHPV, syphilis and gonorrhoea should be considered as risk group and they should be followed with special attention to reduce the rate of ICC in screened patients.

6.3 Association of socioeconomic factors with CIN and ICC

For the last fifty years many studies have been conducted on the social and environmental factors related to development of cervical carcinoma. Bivariate analysis in this study revealed a highly significant association between low level of education and ICC. This association was not nullified by influence of economic status and HPV infection and women of lower education group were three times more likely to develop ICC compared to higher education group. The difference was created by a large number of 'no education' group and very small attendance to secondary education among the women of ICC group. In contrary, Kerala of India, with a considerable reduction of ICC has a high level (>70% of the women population) of secondary education (Varghese et al 1999). Olsen et al (1995) by logistic regression analysis showed that in additional to HPV, only a high number of sexual partners and a low educational level contributed independently to the risk of cervical cancer. When HPV prevalence of all the groups (CIN, ICC and control) were considered together, HRHPV was seen more prevalent among the women with lower education, women who were married in their early years of life and had their 1st childbirth before 16 years of age. All these findings along with the findings of this study supported the role of low educational level as an independent factor in development of cervical cancer. Therefore improvement of education level in the female population has a strong positive effect in reducing the number of ICC and particular attention should be given for improving education level including secondary education of women.

It was interesting to notice that the education level of the women of both CIN and control group were similar. This particular finding indicated that women with better education

been carefully assessed in this population with it's own socio-economic, cultural and religious background. In this conservative society pre-marital sex is not socially accepted and early marriage is almost like a custom in rural Bangladesh.

In this country with poor socioeconomic status, frequently people need to work at a distance from home and have to stay separate from the family. Particularly a considerable number of them work in urban areas due to better job opportunity but is not able to bring their families from the rural areas due to the high living cost in urban areas. People of some profession also need to travel for the nature of their job to different places and stay separate from the family for a long duration. All these may increase the chance of sexual promiscuity and transmission of STD to man and their wives. This study revealed that a greater number of husbands of the women of CIN and ICC group used to travel to different district of the country and stayed separate for a considerable period (> 5 years) than the control group. Therefore, this factor has an important association with ICC. As only a small number of husbands of the ICC and control group were living abroad or travelling abroad (Only five husband of the ICC group and eight of them of the control group), no conclusion could be drawn regarding living status of husband in abroad.

Bivariate analysis showed significant association of higher number of marriage of husband, longer years of sexual activity and staying of husbands away from home with development of CIN and ICC. Apart from that women who married before 18 years of age showed higher risk of developing ICC. When multivariate regression analysis included all these factors along with both risk groups of HPV, the effect of number of husbands marriage, number of years of sexual activity and stay of husband away from home showed independent influence on development of CIN (Table 5.19). On the other hand only early age of first marriage showed seven times higher risk of developing ICC and the effect of other factors did not exist after regression (Table 5.20). This reflected that number of husbands marriage, number of years of sexual activity and travelling status of husband though have some influence in developing CIN, there are other stronger influencing factors as age of 1st marriage and HRHPV for progress of the disease from CIN to ICC. These findings indicate that women with these sexual risk factors need meticulous attention during development of a screening programme.

There is a tendency of arranging marriage in this society once a girl obtain menerchae particularly among the lower income and education group. Study also supported that due to early sexual experience the immature epithelium of the cervix become exposed to different types of infection and spermatozoa at an early age (Coppleson and Reid 1968). Other possible influencing factors include physical and immune status of the female genital tract due to sexual experience in young age (Duncan et al 1990). Shamsuddin et al (1998) also concluded early age at first marriage as the greatest risk factor among the sociodemographic aetiology of pre-malignant cervical abnormalities among women attending a tertiary care hospital in Bangladesh. Other epidemiologic studies also established the association of ICC with early sexual experience (Kjaer 1990, Graaff et al 1977, Martin 1967, Rotkin 1967, Christopherson 1965, Wynder et al 1954). Herrero et al (1990) came to similar conclusion after conducting a large case-control study in four Latin American countries. They mentioned that a period of increased susceptibility to carcinogens during adolescence may be an important determinant of the high incidence of cervical cancer in Latin America. In a hospital-based case-control study in Bankura of West Bengal, where the population and sociocultural factors are very similar to this country, Biswas et al found independent effect of early age at first marriage (Biswas et al 1997).

All these information reflect that prevention of early marriage may play a crucial role in prevention of cervical cancer. Though according to the law of Government of Bangladesh, a girl can not be married before 18 years of age (Repression against Women and Children Act 2000), 92.6 % of the women of this study were already married before the age of 18 years. Marriage registration is helpful in preventing underage marriage. Implementation of the existing marriage act may reduce the incidence of cervical cancer. Health education of parents and population to improve knowledge regarding aetiology of cervical cancer and it's prevention may help in reduction of incidences of marriage of adolescents.

Studies from western countries found a significantly larger proportion of women experienced pre-marital sex and first coitus occurred significantly earlier than the age of first marriage. They concluded that age at first coitus as a more crucial variable than age of first marriage (Kessler 1977, Wynder et al 1954). Therefore the situation is different in

the western world than our society, where the age of first coitus need to be considered separately from the age of first marriage.

Several earlier western studies have led to a conclusion that increasing number of sexual partners were associated with significantly increased risk of ICC (Herrero et al 1990, Kjaer et al 1990, Kessler 1977, Rotkin et al 1967, Wynder et al 1954). In Bangladesh, multiple cohabitation is comparatively less common both among men and women. The mean number of marriage of women was quite low and was almost similar in all three groups and it had no influence on the prevalence of ICC. On the other hand increased number of marriage of husband has some risk of developing ICC among their wives (bivariate analysis). But it is still a less important factor than the western and Latin American countries and not an independent contributing factor in developing ICC.

Women are in a poor social position in this country and frequently they do not know much about their husbands life style. Also, in our social situation, it is very difficult to obtain information on women and their husband's sexual exposure and extra-marital sexual relation. Still five of the women of the ICC group spoken of about extramarital exposure of their husbands which did not happened for the control group. However, this data is very inadequate to comment on influence of extramarital relation of the husbands.

This study revealed that age at first marriage as the most important factor among the sexual factors in developing ICC in this society. This observation is an important finding and verify the association between early age at first marriage and cervical cancer among women with a low rate of sexual promiscuity and define it's role in cervical carcinogenesis among Bangladeshi women.

6.5 Association of reproductive status with CIN and ICC

In this study early age of 1st delivery showed independent influence both in developing ICC and CIN. Several studies also showed a similar finding (Yoshikawa et al 1999, Wynder et al 1954). Coppleson and Reid noticed that malignant transformation of the cervix is most likely to occur when the epithelium is actively undergoing squamous metaplasia, such as in early adolescence and at first pregnancy (Coppleson and Reid 1968). They suggested that DNA viruses might be involved and the spermatozoa

themselves may be mutagenic for the cervix under certain circumstances. Yoshikawa et al (1999) showed early age of first delivery had significant association with cervical cancer even after adjustment of multiple sexual partner. All these information point to an important fact that prevention of early marriage and deferment of 1st delivery at an early age reduces the mutagenic effect of different agents on the cervical epithelium. Therefore, implementation of the marriage act and health education about age of marriage at the national level may significantly reduce the prevalence of ICC.

Wynder et al (1954) revealed that after standardization for age at marriage, the effect of parity does not show any influence in developing cervical cancer. Limited attention has been focused since then on the effect of parity and investigators at that time showed more attention on other risk factors. In 1977 Graaff et al mentioned that there was a consistently smaller proportion of nulliparous married women in the cervical cancer groups than in the control groups. The association of parity with cervical cancer was also raised by Brinton et al (1989). They through a large case-control study in Colombia, Costa Rica, Mexico, and Panama found an association of cervical cancer with high parity and the effect persisted even after adjustment for sexual and socioeconomic variables. They showed that the risks of ICC rose steadily to 5.1 times for those with 14 of more pregnancies and a relationship of risk to multiparity was noticed in all four study countries. This pregnancy related association appeared to be true for parity and not influenced by number of miscarriages or abortions (Brinton et al 1989).

This study found significant and independent association between parity and ICC. However, the independent influence of total number of pregnancy became nullified. The influence of parity remained for the group who had a parity of eight or above. The health policy of Bangladesh government encourages not to have more than two children and to keep family small. This should help gradually decreasing the parity and eliminate parity related cervical cancer from this population in near future.

6.6 Association of contraceptive methods with CIN and ICC

It was difficult to analyse the influence of different contraceptive methods on CIN and ICC because of small sample size. Bivariate analysis showed that increased use of condom among the control group significantly reduced the risk of ICC. Heath education

programme should promote both male and female condoms as an important method of contraception nationwide and this may play important role both by preventing cervical cancer and STD in the society.

OCP is a widely used and popular method of contraception and use of this method has been promoted recently both by health professionals and Government. In this study OCP was the most widely used contraceptive method among all three groups of women (48.1%) and injectable contraceptives were used by 12.2% of them. Among the different contraceptive methods, none of the contraceptive methods was able to show independent influence on development of CIN and ICC.

Several studies found a significant association of OCP with ICC and the risk increased with duration of use (Brinton et al 1989, Brinton et al 1986, La Vecchia et al 1986). On the other hand in a cross sectional follow up study, after adjusting for age, median number of sexual partners, marital status, current smoking, current use of OCP and HRHPV (HC II), only HRHPV and number of sexual partners remain significant. In that study OCP did not show any influence for development of cervical cancer after adjustment of sexual factors (Sellors et al 2003).

Kjellberg et al (2000) in a population based case-control study in Northern Sweden revealed that prolonged period of OCP used (> 5 years) was associated with an increased risk of CIN II and III. But this risk disappeared after adjustment for HPV, smoking and age.

6.7 Limitations of the study

The reported age was not accurate in many cases because the elder members of the community were not always certain of their date of birth. However, the age range was grouped during analysis, which would resolve the anomalies of a few years.

It could not always be possible to brought accurate information regarding land possession status and household earnings. Therefore, information about total monthly income was sometimes not very precise. Again, it was grouped to minimise small scale deviations.

level have improved awareness about their own health and better attendance to receive health service care and this is helpful for diagnosis of precancer state and prevention of reproductive health problems.

Majority of the women in all three groups were housewife, which reflected a poor social and occupational status of the women in Bangladesh. Women, even with a better education level in this society remain as housewife. Occupation of women did not show any association with cervical cancer.

In this study, Husband's occupation also could not show any significant association with cervical cancer. The influence of particular occupation was probably nullified by too much diversity of their occupation and mobility of population to different places for better job opportunity. Therefore, occupation of the couple fail to reveal any association with ICC.

Lower income has independent influence in developing cervical cancer. Prevalence of CIN was three time and ICC was seven times higher among the lower monthly income group. The lower income group may be related to higher incidence of early marriage, high parity, low attendance to the gynaecological clinic and less opportunity of screening. Therefore, all these factors related to poor income make this group vulnerable for the development of cervical cancer. As the income level or education level improves, there is more opportunity of screening, diagnosing and treatment of precancerous conditions and this further helps in declining prevalence of cervical cancer. Thomas et al showed low socioeconomic status as a factor predisposing to persistent oncogenic HPV 16 or 18 infection (Thomas et al 2001).

6.4 Association of sexual behaviour with CIN and ICC

In this study, the mean age of menerchae of all three groups ranged between 12.48-12.79 years and there was no difference among the groups. Wynder et al (1954) also did not show any significant difference of onset of menses between the ICC and control group.

Though the association between sexual behaviour and cervical cancer is well established and the incidence of ICC in Bangladesh is quite high, the role of sexual behaviour has not

Initially attempt was taken to collect information regarding occupation of women and their husband very precisely. Nevertheless due to many varieties of occupation and for the purpose of data analysis we had to arrange it in groups which impaired its preciseness.

A population based study can provide the real picture of the prevalence and epidemiological risk factors. Unfortunately, the proportion of population based research is far smaller than that of the other types of study. This study is also a hospital based research work as it had several advantages as collection of data and specimen were more easier, involvement of less manpower and logistic support.

In this study high and intermediate risk type HPV were grouped as HR HPV and low risk type were grouped as LRHPV and two separate mixture of probes were used. There was no opportunity to identify specific type as type 16, type 18 etc. in this study. Apart from that HPV negative cases could be tested further using HPV E7 type specific PCR and it was not included in the methodology.

It was difficult to assess the influence of different contraceptive methods as the sample size was small to consider all different methods – only the influence of OCP and barrier method could be assessed. Again the influence may be affected by different sexual factors (age of marriage, duration of sexual activity, number of partners and sexual promiscuity). Further study is needed with larger sample size and considering all these factors to assess the independent influence of different contraceptive methods.

Other surrogate biomarkers as HLA-DQw3 and HLA-DR allele which are associated with higher risk of cervical cancer could be assessed as a surrogate biomarker. Frequency of telomerase expression may constitute a biomarker regarding malignant transformation or invasiveness. This study could include the role of this biomarker along with HRHPV.

Male factors were inadequately studied and HPV prevalence of husbands of HPV positive patients could be of interest. Further research is needed to explore male's role in cervical carcinogenesis. HPV DNA prevalence and studies of the natural history of HPV in young men may be of great value. Role of men as vectors of the HPV and their lifetime partners and detection of HPV DNA in the penis of husbands of cancer patients are also surrogate markers of exposure to HPV.

Main obstacle of using HPV test in screening and treatment plan in developing countries are cost and technical requirements. The current HC II test costs in USA is about US$22 per test, and in Bangladesh it may be about 1000 Taka. The test will be more helpful for developing screening protocols utilizing HPV testing if the cost can be reduced further.

Cervical cancer is preventable and both primary and secondary prevention should be practiced in this low socioeconomic background. For primary prevention behaviour modification through women empowerment and consciousness about their own reproductive health is important. Prevention and reduction of other potential co-factors as high parity, early marriage and childbirth is also necessary. Improvement of economic condition and education level will have positive role in prevention of cervical cancer. Primary prevention of cervical cancer through preventing HPV infection by prophylactic vaccines against HPV may contribute cancer prevalence and and vaccine is in the process of development

Worldwide interest is growing in the potential for HPV testing as a part of cervical screening or treatment strategies. In this country with low resource, it may be used as an adjunct to direct visual inspection (DVI) or VIA to develop a programme for cervical cancer screening. These strategies that incorporate DVI or HPV DNA testing, and avoid colposcopy may offer attractive alternatives to cytology-based screening programs in developing countries. Further population based study is needed to explore the sensitivity and specificity of these strategies in this population.

CHAPTER 7

CONCLUSION

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High and intermediate risk HPV infection was found to be the single and strongest determinant for development of cervical carcinoma and its precursors in Bangladesh. The prevalence of HPV infection among the control population is low. Below average economic condition, primary and no formal education, early age of 1st marriage, intercourse and childbirth also increase the risk of cervical cancer.

Improvement of economic status of a family and education level of the female population have a strong positive effect in reducing the number of cervical cancer cases. If the government is stricter about implementation of laws preventing early marriage, it may play a crucial role in prevention of cervical cancer. Spread of awareness regarding natural history of cervical cancer, importance of examination of cervix and participation of women in cervical cancer screening programme demand particular attention.

New guidelines for cervical cancer screening strategies can be developed from the information obtained from this study. In Bangladesh, secondary prevention by hospital-based screening programme is at a very preliminary stage of development, and population screening is just being considered. Cytology based screening programme (Pap smear) will identify a large number of abnormal and indeterminate smear. This is particularly a problem for a country like Bangladesh where facilities and expertise for colposcopic examination are inadequate. Presently colposcopy is less available, expensive and unable to support cytology based screening programme. HPV DNA test may be used to reduce the number of colposcopy. Reduction of number of colposcopy will also reduce the transport cost and family interference and anxiety. These factors are important for implementation of cervical cancer screening at the national level. Since the rate of maternal mortality is very high, the Government of Bangladesh needs a less expensive cervical cancer screening programme and HPV test can be used as an adjunct with direct visual inspection or VIA.

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APPENDICES

A.1: Questionnaire

Association of Human Papillomavirus with Cervical Intraepithelial Neoplasia and Cancer in Bangladeshi Population

Name : Age:

Husbands name :

Address (Postal) : .....

.....

Date of Cervical Smear:

1. Occupation (HW = 1, Service holder = 2, Labour = 3, Salesman = 4, Tearcher = 5)..... ☐
2. Husband's Occupation (Service Holder = 1, Business man = 2, Labour = 3,
   Driver/Conductor = 4, Teacher/Headmaster =5, Died/Divorced = 7,
   Disabled = 8, Retired = 9, Farmer = 10, Jobless = 11) Others – please mention..... ☐
3. Education (No education =1, Primary=2, Secondary =3,
   Higher = 4, Do not know =9)..... ☐
4. Husband`s Education (no education =1, Primary =2, Secondary =3,
   Higher= 4,Do no`t know =9)..... ☐
5. Socioeconomic status
   (Poor = 3000=1, Lower middle class =3000-80000=2
   Higher middle class =3000-12000=3, Rich=Tk>12000=4)..... ☐
6. Menstrual cycle (Regular=1, Irregular=2, Menopause = 3,
   Amenorrhoea due to Inj. = 4 Ameno due to other = 5)..... ☐
7. LMP ..... ☐☐☐
8. Duration of menstruation (In days)..... ☐☐
9. Amount of bleeding (Normal=1, Scanty = 2 , Excessive=3)..... ☐
10. Postcoital bleeding (No=1, yes=2)..... ☐
11. Intermenstrual bleeding (No=1, Yes=2)..... ☐
12. Vaginal discharge(Normal=1, excessive =2, blood stained=3, foul smell=4)..... ☐
13. Contraception (No=1, withdrawal method=2, safe period = 3, barrier = 4, OCP=5
    Injectable=6, Norplant=7, IUD=8, Vasectomy=9, tubectomy=10, other specify.)..... ☐
14. H/O previous c.smear (No =1, Yes=2) If yes date and findings..... ☐
15. Para..... ☐☐

16. No of total pregnancy..... ☐☐
17. Age at the end of 1st pregnancy..... ☐☐
18. Age of 1st intercourse..... ☐☐
19. Number of marriage..... ☐
20. Number of marriage of Husband ..... ☐
21. Husband living or travelling abroad : (Living together =1,
Living abroad =2, travelling abroad =3, Husband died = 4, Husband separated =5)..... ☐
22. How many years sexually active..... ☐☐
23. Per abdominal..... ☐
24. Vulva ..... ☐
25. Vaginal discharge (Normal=1, Excessive=2, blood stained =3, foul smell=4)..... ☐
26. CX (Healthy =1, hypertrophied=2, erosion =3, congested = 4,
polyp=5, growth=6, bleeds on touch=7, other specify)..... ☐
27. Uterus (Normal size=1, Bulky =2, Uterine prolapse = 3)..... ☐
28. Adnexa (Normal =1, enlarged = 2, Tender = 3)..... ☐
29. C. Smear report (Inadequate + 1, Normal = 2, Inflammatory = 3,
mild dyskariosis = 4, Mod dyrkariosis = 5, Severe dyrkarosis =6,
Atypical cell = 7, Mild atypia = 8, Koilocytosis = 9, In sq-cell ca=10,
trichomoniasis + N C. Smear = 11, atropic Smear =12, Unsatisfectory = 13)..... ☐☐
30. Management (No step needed, advice to repeat 3yr = 1, Repeat C. S = 2,
Colposcopy = 3, Antibiotics + repeat = 4, Advice TAH = 5, Cone biopsy,= 6, ☐
31.Colposcope findings (Chronic cervicitis / NAD =1, NAD =2,
CIN I = 3, CIN II = 4, CIN III = 5..... ☐
32. HPE Report (Normal, Chronic cervicitis = 1, Mild dyskariosis = 3
Mod dyskariosis = 4, Severe dyskariosis = 5)..... ☐
33. Further Management (Cauterization of Cx = 1, Cone biopsy = 2, TAH = 3..... ☐
34. Relegion (Muslim = 1, Hindu = 2, Other = 3)..... ☐
35. Histopathology report..... ☐
36. HPV DNA report..... ☐
37. Gonococcal DNA report..... ☐
38. HSV type II – serology report..... ☐
39. *T Pallidum* - serology report..... ☐

A.2: Urban area

'Urban area' is considered as described by Bangladesh Population Census of 1991. The mentioned Census described 'Urban area' as developed areas around an identifiable central place where amenities like asphalt roads, telephone, electricity, gas, water supply, sewerage, sanitation etc usually exist, which are densely populated and majority of the population are non-agricultural and where community sense is well developed. Three different types of urban areas have been defined (Statistical metropolitan areas, Municipality area and other urban area). Other urban areas included Thana head quarters and the development centres which have urban characteristics.

A.3: FIGO classification

Stage 0 Carcinoma *in situ*, CIN3

Stage I The carcinoma is strictly confined to the cervix

Stage II Cervical carcinoma invades beyond the uterus, but not to the pelvic wall or to the lower third of the vagina.

Stage III The carcinoma has extended to the pelvic wall. The tumour involves the lower third of vagina.

Stage IV The carcinoma extends beyond the true true pelvis, or involves the mucosa of the bladder or rectum.

A.4: Colposcopical examination

The Colposcope is a binocular magnifying instrument that examines the surface features and vascular pattern of the cervical and vaginal epithelia. It magnifies the view of the surface of the cervix and, in most patients, of the endocervical canal, and allows precise delineation of the size and distribution of the neoplastic epithelium. During colposcopy, the women had adequate counseling regarding the smear report, the purpose of colposcope, the procedure of colposcopical examination, possible reports and further management. The patient was placed in lithotomy position and a cuscose bivulved speculum was passed into the vagina to expose the cervix. The cervix was gently swabbed with saline to remove excessive discharge and the appearance of the cervix was observed with the colposcope. Dilute acetic acid (5%) was applied with a cotton wool swab and the degree of acetowhite change and the clarity of its demarcation from normal epithelium were noted. At least two colposcopy directed biopsies were taken from the most severely affected area and sent for histopathological examination of BSMMU for examination and interpretation.

A.5: Cervical sampler

Cervical sampler used for collection and transport of cervical specimens were imported from Digene corporation of USA. Each cervical sampler contains one cervical brush and a tube containing one ml Specimen transport medium (STM). The STM contained a preservative to retard bacterial growth and to retain the integrity of DNA. It can not preserve viability of organisms or cells. Digene cervical sampler is stored at 15-30°C until the expiration date on the pouch label.

A.6: Equipment required for HC II test

DML 2000 ™ Luminometer

Rotary shaker with adjustable speed setting

Wash apparatus

Specimen collection tube rack (to fit specimen collection tubes)

PC system (486 PC, keyboard, monitor, Windows 95 and excel 7.0 software, and printer)

65 $\pm 2^0$C water bath of sufficient size to hold specimen racks

Vortex mixer with cup attachment

Single channel micropipettor; variable settings for 20-200 µl volumes

Repeating positive displacement pipettor such as eppendorf or equivalent

8-channel pipettor

Timer

A.7: Reagents and materials provided for HC II test

(For 96 Tests - 42 or 87 specimens)

Indicator dye :	Contains sodium azide
Denaturation Reagent :	Dilute sodium hydroxide (NaOH) solution
Probe Diluent :	Buffered solution with sodium azide
HPV Probe A :	HPV 6/11/42/43/44 RNA probe cocktail in buffered solution (green cap)
HPV Probe B :	HPV 16/18/31/33/35/39/ 45/51/52/56/58/59/68 RNA probe cocktail in buffered solution (red cap)
Negative Control:	Carrier DNA in STM with sodium azide
Calibrator A:	1.0 pg/ml constructed HPV 11 DNA and carrier DNA in STM with sodium azide
Calibrator B:	1.0 pg/ml constructed HPV 16 DNA and carrier DNA in STM with sodium azide
Capture Microplate:	coated with anti-RNA:DNA hybrid antibodies
Detection Reagent 1:	Alkaline phosphatase-conjugated antibodies to RNA:DNA hybrids in buffered solution with sodium azide
Detection Reagent 2:	CDP-Star ™ with Emerald II (chemiluminescent substrate).

Wash buffer Concentrate: Contains sodium azide

A.8: Reagents preparation and Storage for HC II test

Upon receipt, the kits were stored at 2-8°C. The kits were used before the expiration date. All reagents are ready-to-use except denaturation reagent, HPV Probes A and B, and wash buffer.

To test specimens for the presence of any of the 18 HPV types, a CPC Method has been provided. To test using this option, a CPC must be prepared by mixing diluted Probe A cocktail and diluted Probe B cocktail together in advance of performing the Digene HPV Test. The Two-Probe method uses separate Probe A and B cocktails.

Preparation method of denaturation reagent

Five drops of indicator dye was added to the bottle of denaturation reagent and mixed thoroughly. The denaturation reagent should be a uniform, dark purple color.

Once prepared, the denaturation reagent was stable for three months when stored at 2-8°C. If the color fades, 5 drops of indicator dye was added and it was mixed thoroughly before use.

Preparation method of HPV Probe A cocktail

(Prepared from HPV Probe A and probe diluent reagents).

The Probe A cocktail was prepared during specimen denaturation procedure.

(NOTE: Probe diluent is viscous. Adequate care was taken to ensure thorough mixing during preparing HPV probes. A visible vortex was always noted in the liquid during the mixing step. Incomplete mixing may result in reduced signal. The smallest number of wells recommended for each use is 24. If fewer than 24 wells per run are desired, the total number of tests per kit may be reduced due to limited probe and probe diluent volumes.)

The vial of HPV Probe A was centrifuged to bring liquid to bottom of vial. It was gently tabbed to mix. The amount of Probe mix required (25 µl/ test) was determined (volumes listed below). Extra Probe mix was made to account for the volume which may be lost in the pipette tips or on the side of the vial..

The required amount of probe diluent was transferred to a new disposable container. A dilution of 1:25 was made from HPV Probe A in probe diluent to prepare probe mix.

No. of Tests/Strips	Volume Probe Diluent*	Volume Probe*
96/12	3.5 ml	140.0 µl
73/9	2.6 ml	105.0 µl
48/6	1.75 ml	70.0 µl
24/3	0.875 ml	35.0 µl
1	0.040 ml	1.6 µl

*These values include the recommended extra volume.

HPV Probe A was pipetted into probe diluent by placing the pipette tip against the inner wall of the tube just above the meniscus and expelling the contents. The pipette tip was not immersed into probe diluent. It was vortex for at least 5 seconds at maximum speed to mix thoroughly. It was labelled as "HPV Probe A Cocktail". Unused probe mix was discarded.

Preparation method of HPV Probe B cocktail

(Prepared from HPV Probe B and probe diluent reagents).

It was prepare as HPV Probe A mentioned above. It was labelled as "HPV Probe B Cocktail".

Preparation method of HPV Combined Probe cocktail (CPC)

Probe A and Probe B cocktails were prepared seperately as described above. The entire contents of diluted Prove A Cocktail was added to the tube of diluted Probe B cocktail. The content was mixed thoroughly by vortexing for at least 5 seconds at maximum speed. The content was labelled as "Combined Probe Cocktail".

Preparation method of wash buffer

(The wash buffer was prepared during capture step)

100 ml wash buffer concentrate was diluted with 2.9 L of distilled or deionized water and it was mixed well (final volume 3 L). The container was sealed to prevent contamination or evaporation.

The wash buffer is stable for three months at 2-25°C after preparation. If the wash buffer has been refrigerated, it was equilibrated to 20-25°C before use.

It is recommended that the wash apparatus and tubing be cleaned with bleach and rinsed thoroughly with deionized water once every three months to prevent possible contamination from alkaline phosphatase present in bacteria and molds.

A.9: Quality Control for HC II test

The negative control and positive calibrator were run in triplicate with each test run. The negative control and positive calibrator results should show a coefficient of variation (%CV) of ≤ 25%. If the % CV is > 25%, the control or calibrator value with a RLU value furthest from the mean was discarded as an outlier, and the mean using the remaining two values was recalculated. If the difference between the mean and each of the two values was ≤ 25%, the next step was continued; otherwise, the assay was invalid and was repeated.

The mean of the negative control results were ≤ 250 RLUs. If the mean of the negative control was > 250 RLUs, the assay was considered invalid and was repeated.

The Positive calibrator mean (PC8) and negative control mean (NC8) results were used to calculate the CA8 / NC8 ratio for each probe. These ratios must meet the following criteria to validate the assay before the specimen results can be interpreted:

CPC METHOD	TWO-PROBE METHOD
Assay validation Acceptable ranges	Assay validation Acceptable ranges
CAξ / NC8 ≥ 2.0 CBξ / NC8 ≥ 2.0	CAξ / NC8 ≥2.0 CBξ / NC8 ≥ 2.0

The appropriate PC8 / NC8 ratios for each of the probe set calibrators were calculated. Whenever the ratios were ≥ 2.0 for CPC or ≥ 2.0 for the two probe method, the next step of the process was proceeded. If any of the ratios failed, the assay was invalid for that specific probe set and was repeated.

A.10: Limitations of HPV test by HC II

1. In Vitro test.
2. The Digene HPV test procedure, quality control and the interpretation of specimen results must be followed closely to obtain reliable test results.
3. It is important to pipette the exact reagent volume indicated and to mix well after each reagent addition. Failure to do so could result in erroneous test results. Ensuring that the noted color changes occur will help to confirm whether these conditions have been met.
4. A negative result does not exclude the possibility of HPV infection since very low levels of infection or sampling error may cause a false negative result.
5. The Digene HPV test can only be used with cervical specimens collected using the Digene cervical sampler, Digene specimen collection kit, Digene specimen transport medium or cervical specimens collected using a broom-type collection device and placed in Cytyc ThinPrep Pap Test PreservCyt Solution. Biopsy specimens may be assayed only if they are placed immediately in Digene specimen transport medium and stored at -20^{oC} until assayed.
6. The Digene HPV test distinguishes between two groups of HPV types: HPVs 6/11/42/43/44 and 16/18/31/33/35/39/45/51/52/56/58/59/68. It can not distinguish among the viral types within these groups.
7. If the RLU/ PC8 ratio of a specimen is close to but less than 1.0 and HPV infection is suspected, alternate testing methods and /or a repeat specimen should be considered.

A.11: Serological detection of syphilis

Procedure :

The samples and reagents were allowed to reach room temperature and care was taken so that samples and all reagents were fully resuspended before use.

Screening Procedure

The diluent was dispensed into the microtitration plate as follows:

In row 1 - 25 µl (1 drop)

In row 2 - 100 µl (4 drops)

In row 3 and 4 - 25 µl (1 drops)

One drop of each sample was added into each well in row 1 containing 25 µl of diluent. Using a microtitration diluter (or a pipette set to 25 µl) row 1 was mixed and 1 volume was transferred to row 2. It was mixed and 1 volume of row 2 was transferred to row 3. The content of row 3 was mixed and 1 volume from row 3 was discarded. A second volume from row 2 was transferred to row 4, the content was mixed and 1 volume from row 4 was discarded.

One drop (75 µl) of Test Cells was added to row 4 using the attached cell dropper and one drop (75 µl) of Control Cells was added to row 3 using the same. The plate was tapped gently to mix. This resulted in final serum dilutions in row 3 and 4 to 1/80.

The plate was covered and allowed to stand for 45-60 minutes (alternatively the plates can be left overnight).

The agglutination patterns were examined.

Results and Interpretation

Agglutinated cells form an even layer over the bottom of the well. Non-agglutinated cells form a compact button in the centre of the well. Weakly agglutinated cells form a characteristic ring pattern. Agglutination of the Test Cells but not the Control Cells indicates the presence of specific antibody to T. pallidum. Absence of agglutination indicates that antibody is below the limit of detection of the system. Do not use the Control Cell patterns as an indication of a negative result since they give a more compact button of cells.

Agglutination of the Control Cells as well as the Test Cells indicates the presence of anti-cell antibody. In this even the test is not valid and should be repeated. Should the test not be valid the test should be repeated after first performing and absorption of the test serum. To achieve this, dilute the test serum ¼ with Control Cells and allow to stand at room temperature for 45-60 minutes. After centrifuging the sample (1000rpm/5 mins) dilute the supernatent 1/5 in Diluent. Test this dilution directly, without any further dilution, using Test and Control Cell suspensions. A confirmatory FTA-ABS test is also recommended.

A.12: Serological detection of HSV 2

Assay procedure

All the kit components (Omega) and the test samples were brought to room temperature before use.

A Data Sheet was prepared to identify the individual wells for each sample and the controls. One High Positive Control, two Low Positive Controls and one Negative Control were included in each run. Unused strips were resealed in the foil bag containing the desiccant using the resealing zip-lock, before being replaced at 2°C to 8°C for storage.

The samples were diluted to 1/20 using the Sample Diluent (e.g. 10µl of sample+190µl of Sample Diluent). The Controls included in the kit were already prediluted and were ready to use. So the controls were not diluted.

100µl of diluted sample or Control were added to the appropriate well on the Microtitration Plate. The plate were gently shaken for 5 seconds.

The plate were incubated for 20 minutes at room temperature (20°C).

At the end of the incubation period, the contents of the wells were discard by turning the plate rapidly upside down over a Biohazard container, then, the plate was striked (still upside down) against an absorbent paper or paper towel. Adequate amount of disinfectant in the biohazard container was ensured.

The Plate was washed 3 times.

100µl of Conjugate was dispensed into each well. It was gently shaken for 5 seconds. It was incubated for 20 minutes at room temperature (20°C to 25°C). At the end of the incubation period, the contents was discarded and washed. 100µl substrate solution was added to each well. It was gently shaken for 5 seconds. The reaction was allowed to develop in the dark for 10 minutes at room temperature (20°C to 25°C). The reaction was stopped by adding 100µl stop solution to each well. This produced a colour change from blue to yellow in wells containing enzyme that indicates the presence of anti-HSV II IgG antibodies. The absorbency of each well at 450nm, was measured immediately after stopping the reaction.

Calculation and Interpretation of Results

The average Optical Density (OD) for each test and control sera was obtained during the test run. The results were calculated. The average OD of the Low Positive Control was calculated. This was the cut-off value of the assay. The sample OD was divided by the value obtained above. A ratio greater than 1.1 indicated a positive sample.

A 13 : Assay validation of DNA detection of HRHPV Probe

Assay:	Digene HPV Probe B	Type:	Single	Area:	A1..D7

Assay is valid

Operator:	ashrafunnessa	Kit Lot #	2120kx01	Exp. Date:	7/30/02	Room Temp:	25

Comment: study

Software rev: 1.1.3

Replicates:		Negative		Positive		QC	
Blanks:	0	Cal:	3	Cal:	3	Ctrls	1
Specimens:	1						

Number of QC Control types required for assay validation: 0

Assay validation: **Discard Outliers:** **Yes**

Max. NC:	250	*Min. NC:*	10	*Max. %CV:*	25
Max. PC:	N/A	*Min. PC:*	N/A	*Max. %CV:*	25
Control Ratio -	PC/NC	*Max:*	20	*Min:*	2
Mean NC =	127.00	%CV =	8.90	**%CV - valid**	**Mean NC valid**
Mean PC =	301.00	%CV =	13.15	**%CV - valid**	**Mean PC N/A**
Ratio =	2.37	**Ratio:**	**valid**		

Cutoff Calculation:

Cutoff Factor: 1 *Cutoff* = Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF* = 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF* = 1

Values: **Pos. Cutoff** ≥ 301.00 **Neg. Cutoff** < 301.00 301.00 ≤ **Equivocal** < 301.00

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ **Date:** _____

A 14: DNA deletion report of HRHPV from ICC Specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene HPV Probe B	A1 D7	ashrafunnessa	2120k401	Yes	Yes	301 00	301 00

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	NC	Chr32	Chr40	Chr48	Chr56	Chr64	Chr72					
RLU	75	508697	362936	526838	19541	360206	725754					
Ratio		2022 24	1205 76	1750 29	61 93	1183 47	2411 14					
Result	<outlier rejected>	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +					
B												
ID	NC	Chr33	Chr41	Chr49	Chr57	Chr65	Chr73					
RLU	119	530199	109367	23157	10853	35005	430776					
Ratio		1761 45	363 34	76 93	36 05	116 29	1431 14					
Result		High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +					
C												
ID	NC	Chr34	Chr42	Chr50	Chr58	Chr66	Chr74					
RLU	135	727964	462221	599328	9889	517262	145095					
Ratio		2418 48	1535 81	1991 12	32 85	1718 47	482 04					
Result		High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +					
D												
ID	PC-B	Chr35	Chr43	Chr51	Chr59	Chr67	Chr75					
RLU	525	525710	5437	252233	198529	600686	170095					
Ratio		1748 54	18 06	837 98	659 89	1995 63	565 09					
Result	<outlier rejected>	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +					
E												
ID	PC-B	Chr36	Chr44	Chr52	Chr60	Chr68						
RLU	273	12305	77707	555084	789535	418115						
Ratio		40 88	258 16	1844 13	2623 37	1389 08						
Result		High Risk +	High Risk +	High Risk +	High Risk +	High Risk +						
F												
ID	PC-B	Chr37	Chr45	Chr53	Chr61	Chr69						
RLU	329	589913	611253	586230	534849	187						
Ratio		1959 84	2030 74	1947 60	1776 90	0 62						
Result		High Risk +	High Risk +	High Risk +	High Risk +	--						
G												
ID	Chr30	Chr38	Chr46	Chr54	Chr62	Chr70						
RLU	261901	411623	70989	20031	88239	267886						
Ratio	870 10	1367 51	235 84	66 54	293 15	889 98						
Result	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +						
H												
ID	Chr31	Chr39	Chr47	Chr55	Chr63	Chr71						
RLU	378267	231584	233679	88705	61889	9851						
Ratio	1256 70	769 38	776 34	294 70	205 61	32 72						
Result	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +	High Risk +						

A 15: Assay validation of DNA detection of LRHPV Probe

Assay:	Digene HPV Probe A	**Type:**	Single	**Area:**	A1. D7

Assay is valid

Operator:	ashrafunnessa	**Kit Lot #**	2120kx01	**Exp. Date:**	7/30/02
Room Temp:	25				
Comment:	study			**Software rev:**	1.1.3

Replicates:					
Blanks: 0	Negative Cal: 3	Positive Cal: 3	QC Ctrls: 1		
Specimens: 1					

Number of QC Control types required for assay validation: 0

Assay validation:		**Discard Outliers:**	**Yes**
Max. NC: 250	*Min. NC:* 10	*Max. %CV:* 25	
Max. PC: N/A	*Min. PC:* N/A	*Max. %CV:* 25	
Control Ratio - PC/NC	*Max:* 20	*Min:* 2	
Mean NC = 48.66	%CV = 24.07	**%CV - valid**	**Mean NC valid**
Mean PC = 216.66	%CV = 10.69	**%CV - valid**	**Mean PC N/A**
Ratio = 4.45	**Ratio: valid**		

Cutoff Calculation:

Cutoff Factor: 1 *Cutoff =* Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF =* 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF =* 1

RLU

Values: **Pos. Cutoff ≥** 216.66 **Neg. Cutoff <** 216.66 216.66 **≤ Equivocal <** 216.66

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ **Date:** _____

A 16 : DNA detection report of LRHPV from Icc Specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene HPV Probe A	A1 .O7	ashrafunnessa	2120kx01	Yes	Yes	216 66	216 66

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	NC	Clr32	Clr40	Clr48	C r56	Clr64	Clr72					
RLU	46	46	140	60	46	234	72					
Ratio		0 21	0 64	0 27	0 21	1 08	0 33					
Result		--	--	--	--	Low Risk +	--					
B												
ID	NC	Clr33	Clr41	Clr49	C r57	Clr65	Clr73					
RLU	44	84	40	214	40	258	66					
Ratio		0 38	0 18	0 98	0 18	1 19	0 30					
Result		--	--	--	--	Low Risk +						
C												
ID	NC	Clr34	Clr42	Clr50	Clr58	Clr66	Clr74					
RLU	62	130	76	82	36	192	50					
Ratio		0 60	0 35	0 37	0 16	0 88	0 23					
Result		--	--	--	--	--	--					
D												
ID	PC-A	Clr35	Clr43	Clr51	Clr59	Clr67	Clr75					
RLU	192	144	44	54	42	143	58					
Ratio		0 66	0 20	0 24	0 19	0 66	0 26					
Result		--	--	--	--	--	--					
E												
ID	PC-A	Clr36	Clr44	Clr52	Clr60	Clr68						
RLU	238	50	48	64	76	1112						
Ratio		0 23	0 22	0 29	0 35	5 13						
Result		--	--	--	--	Low Risk +						
F												
ID	PC-A	Clr37	Clr45	Clr53	Clr61	Clr69						
RLU	220	118	76	50	94	98						
Ratio		0 54	0 35	0 23	0 43	0 45						
Result		--	--	--	--	--						
G												
ID	Clr30	Clr38	Clr46	Clr54	Clr62	Clr70						
RLU	50	88	86	52	50	78						
Ratio	0 23	0 40	0 39	0 24	0 23	0 36						
Result	--	--	--	--	--	--						
H												
ID	Clr31	Clr39	Clr47	Clr55	Clr63	Clr71						
RLU	148	52	104	40	42	44872						
Ratio	0 68	0 24	0 48	0 18	0 19	207 10						
Result	--	--	--	--	--	Low Risk +						

A 17: Assay validation of DNA detection of HRHPV Probe

Assay: Digene HPV Probe B **Type:** Single **Area:** A1..H6

Assay is valid

Operator: ashrafunnessa **Kit Lot #** 2003kx01 **Exp. Date:** 5/31/02 **Room Temp:** 25

Comment: study **Software rev:** 1.1.3

Replicates:

	Negative	Positive	QC
Blanks: 0	Cal: 3	Cal: 3	Ctrls 1
Specimens: 1			

Number of QC Control types required for assay validation: 0

Assay validation: **Discard Outliers:** Yes

Max. NC: 250	*Min. NC:* 10	*Max. %CV:* 25	
Max. PC: N/A	*Min. PC:* N/A	*Max. %CV:* 25	
Control Ratio - PC/NC	*Max:* 20	*Min:* 2	
Mean NC = 223.00	%CV = 10.87	**%CV - valid**	**Mean NC valid**
Mean PC = 2041.00	%CV = 7.76	**%CV - valid**	**Mean PC N/A**
Ratio = 9.15	**Ratio: valid**		

Cutoff Calculation:

Cutoff Factor: 1 *Cutoff =* Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF* = 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF* = 1

[redacted]alues: **Pos. Cutoff ≥** 2041.00 **Neg. Cutoff <** 2041.00 2041.00 **≤ Equivocal <** 2041.00

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ **Date:** _____

A 18: DNA detection report of HRHPV from CIN Specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene HPV Probe B	A1 H6	ashrafunnessa	2003kx0*	Yes	Yes	2041.00	2041.00

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	N	dhr3	dhr11	dhr19	dhr27	dhr35						
RLU	197	203	343549	4009	4343	51919						
Ratio		0.09	158.32	1.96	2.12	254.38						
Result		--	High Risk +	High Risk +	High Risk +	High Risk +						
B												
ID	NC	dhr4	dhr12	dhr20	dhr28	dhr36						
RLU	227	18881	5171	2541	4075	5083						
Ratio		9.25	2.53	1.24	1.99	2.48						
Result		High Risk +	High Risk +	High Risk +	High Risk +	High Risk +						
C												
ID	NC	dhr5	dhr13	dhr21	dhr29	dhr37						
RLU	245	139	454761	3219	3725	5195						
Ratio		0.06	222.82	1.57	1.82	2.54						
Result		--	High Risk +	High Risk +	High Risk +	High Risk +						
D												
ID	PC-B	dhr6	dhr14	dhr22	dhr30	dhr38						
RLU	2153	481	1989	3919	15959	6557						
Ratio		0.23	0.96	1.92	7.81	3.21						
Result		--	--	High Risk +	High Risk +	High Risk +						
E												
ID	PC-B	dhr7	dhr15	dhr23	dhr31	dhr39						
RLU	1929	1143977	797	2963	4643	5661						
Ratio		560.49	0.39	1.45	2.27	2.87						
Result		High Risk +	--	High Risk +	High Risk +	High Risk +						
F												
ID	PC-B	dhr8	dhr16	dhr24	dhr32	dhr40						
RLU	1209	369	737	1831	143861	5539						
Ratio		0.18	0.36	0.79	70.48	2.71						
Result	<outlier rejected>	--	--	--	High Risk +	High Risk +						
G												
ID	dhr1	dhr9	dhr17	dhr25	dhr33	dhr41						
RLU	307	3977	1989	4379	7231	5983						
Ratio	0.15	1.94	0.97	2.14	3.54	2.93						
Result	--	High Risk +	--	High Risk +	High Risk +	High Risk +						
H												
ID	dhr2	dhr10	dhr18	dhr26	dhr34	dhr42						
RLU	185	701	5433	1175	4369	6079						
Ratio	0.09	0.34	2.66	0.57	2.14	2.97						
Result	--	--	High Risk +	--	High Risk +	High Risk +						

A 19 : Assay validation of DNA detection of LRHPV Probe

Assay:	Digene HPV Probe A	**Type:**	Single	**Area:**	A1..H6

Assay is valid

Operator:	ashrafunnessa	**Kit Lot #**	2003kx01	**Exp. Date:**	5/31/02	**Room Temp:**	25
Comment:	study			**Software rev:**	1.1.3		

Replicates:				
	Negative	Positive	QC	
Blanks: 0	Cal: 3	Cal: 3	Ctrls: 1	
Specimens: 1				

Number of QC Control types required for assay validation: 0

Assay validation:		**Discard Outliers:**	**Yes**
Max. NC: 250	*Min. NC:* 10	*Max. %CV:* 25	
Max. PC: N/A	*Min. PC:* N/A	*Max. %CV:* 25	
Control Ratio - PC/NC	*Max:* 20	*Min:* 2	
Mean NC = 187.00	%CV = 15.12	**%CV - valid**	**Mean NC valid**
Mean PC = 739.66	%CV = 10.17	**%CV - valid**	**Mean PC N/A**
Ratio = 3.95	**Ratio: valid**		

Cutoff Calculation:

Cutoff Factor: 1 *Cutoff* = Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF* = 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF* = 1

RLU Values: **Pos. Cutoff ≥** 739.66 **Neg. Cutoff <** 739.66 739.66 **≤ Equivocal <** 739.66

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ **Date:** _____

A 20 : DNA deletion report of LRHPV from CIN Specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene HPV Probe A	A1.H6	ashrafunnesa	2003kx01	Yes	Yes	739 66	739 66

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	[illegible]	hpvlrD3	hpvlrD11	hpvlrD19	hpvlrD27	hpvlrD35						
RLU	835	207	323	219	401	303						
Ratio		0 27	0 43	0 29	0 54	0 40						
Result	<control rejected>	--	--	--	--	--						
B												
ID	M	hpvlrD4	hpvlrD12	hpvlrD20	hpvlrD28	hpvlrD36						
RLU	207	167	243	157	149	1683						
Ratio		0 22	0 32	0 21	0 20	2 27						
Result	--	--	--	--	--	Low Risk +						
C												
ID	N	hpvlrD5	hpvlrD13	hpvlrD21	hpvlrD29	hpvlrD37						
RLU	167	109	385	651	141	511						
Ratio		0 14	0 49	0 88	0 19	0 69						
Res.		--	--	--	--	--						
D												
ID	PC-A	hpvlrD6	hpvlrD14	hpvlrD22	hpvlrD30	hpvlrD38						
P	799	209	129	261	221	341						
R		0 26	0 17	0 35	0 29	0 46						
[illegible]ult		--	--	--	--	--						
J	PC-A	hpvlrD7	hpvlrD15	hpvlrD23	hpvlrD31	hpvlrD39						
RLU	655	1877	151	839	227	261						
Ratio		2 87	0 20	1 13	0 30	0 35						
Result		Low Risk +	--	Low Risk +	--	--						
F												
ID	PC-A	hpvlrD8	hpvlrD16	hpvlrD24	hpvlrD32	hpvlrD40						
RLU	765	423	151	247	199	483						
Ratio		0 57	0 20	0 33	0 26	0 62						
Result	--	--	--	--	--	--						
G												
ID	hpvlrD1	hpvlrD9	hpvlrD17	hpvlrD25	hpvlrD33	hpvlrD41						
RLU	107	311	151	379	105	183						
Ratio	0 14	0 42	0 20	0 51	0 14	0 24						
Result	--	--	--	--	--	--						
H												
ID	hpvlrD2	hpvlrD10	hpvlrD18	hpvlrD26	hpvlrD34	hpvlrD42						
RLU	285	193	95	161	2785	213						
Ratio	0 38	0 26	0 12	0 21	3 73	0 28						
Result	--	--	--	--	Low Risk +	--						

A 21 : Assay validation of DNA detection of Combined HRHPV & LRHPV Probe

Assay:	Digene HPV CPC	Type:	Combined	Area:	A1..H12

Assay is valid

Operator:	ashrafunnessa	Kit Lot #	2003kx01	Exp. Date:	5/31/02	Room Temp:	25
Comment:	study			Software rev:	1.1.3		

Replicates:		Negative		Positive		QC	
Blanks	0	Cal:	3	Cal 1:	3	Ctrls:	1
Specimens	1			Cal 2:	3		

Number of QC Control types required for assay validation: 0

Assay validation: **Discard Outliers:** **Yes**

Max NC	250	*Min. NC:*	10	*Max. %CV:*	25
Max PC	N/A	*Min. PC:*	N/A	*Max. %CV:*	25
Control Ratio - PC/NC		*Max:*	20	*Min:*	2

Mean NC =	175 66	%CV =	23.99	**%CV - valid**	**Mean NC valid**
Mean PC1 =	410 00	PC1 %CV =	4.48	**%CV - valid**	**Mean PC1 N/A**
Mean PC2 =	438 00	PC2 %CV =	0.96	**%CV - valid**	**Mean PC2 N/A**
Mean Comb PC =	424 00	Combined %CV =	4.59	**Comb. %CV - valid**	**Comb. Mean PC N/A**
Ratio =	2 41	**Ratio:**	**valid**		

Cutoff Calculation:

Cutoff Factor *Cutoff* = Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF* = 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF* = 1

RLU values: **Pos. Cutoff ≥** 424.00 **Neg. Cutoff <** 424.00 424.00 **≤ Equivocal <** 424.00

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ Date: _____

22: DNA deléction report of Combined HRHPV & LRHPV from Control Specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene HPV CPC	A1. H12	ashrafunnessa	2003kx01	Yes	Yes	424 00	424 00

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	NC	PC-B	hpvcpcC8	hpvcpcC16	hpvcpcC24	hpvcpcC32	hpvcpcC40	hpvcpcC48	hpvcpcC56	hpvcpcC64	hpvcpcC72	hpvcpcC80
RLU	127	441	131	111	75	107	109	279	89	101	409	183
Ratio			0 30	0 25	0 17	0 25	0 25	0 65	0 20	0 23	0 96	0 38
Result												
B												
ID	NC	hpvcpcC1	hpvcpcC9	hpvcpcC17	hpvcpcC25	hpvcpcC33	hpvcpcC41	hpvcpcC49	hpvcpcC57	hpvcpcC65	hpvcpcC73	hpvcpcC81
RLU	199	119	106	107	109	369	105	89	91	213	249	183
Ratio		0 28	0 25	0 25	0 25	0 87	0 24	0 20	0 21	0 50	0 58	0 38
Result												
C												
ID	NC	hpvcpcC2	hpvcpcC10	hpvcpcC18	hpvcpcC26	hpvcpcC34	hpvcpcC42	hpvcpcC50	hpvcpcC58	hpvcpcC66	hpvcpcC74	hpvcpcC82
RLU	201	141	123	107	99	105	95	131	85	169	95	165
Ratio		0 33	0 29	0 25	0 23	0 24	0 22	0 30	0 20	0 39	0 22	0 34
Result												
D												
ID	PC-A	hpvcpcC3	hpvcpcC11	hpvcpcC19	hpvcpcC27	hpvcpcC35	hpvcpcC43	hpvcpcC51	hpvcpcC59	hpvcpcC67	hpvcpcC75	hpvcpcC83
RLU	423	89	93	1381	227	113	85	93	385	135	119	65
Ratio		0 20	0 21	3 25	0 53	0 26	0 20	0 21	0 90	0 31	0 28	0 24
Result				HPV +								
E												
ID	PC-A	hpvcpcC4	hpvcpcC12	hpvcpcC20	hpvcpcC28	hpvcpcC36	hpvcpcC44	hpvcpcC52	hpvcpcC60	hpvcpcC68	hpvcpcC76	hpvcpcC84
RLU	397	97	93	105	99	115	97	101	101	95	123	155
Ratio		0 22	0 2*	0 24	0 23	0 27	0 22	0 23	0 23	0 22	0 29	0 80
Result												
F												
ID	PC-A	hpvcpcC5	hpvcpcC13	hpvcpcC21	hpvcpcC29	hpvcpcC37	hpvcpcC45	hpvcpcC53	hpvcpcC61	hpvcpcC69	hpvcpcC77	hpvcpcC85
RLU	869	127	111	93	105	101	282*	121	131	161	117	113
Ratio		0 29	0 26	0 21	0 24	0 23	6 65	0 28	0 30	0 37	0 27	26
Result	<outlier rejected (Pos1)>						HPV +					
G												
ID	PC-B	hpvcpcC6	hpvcpcC14	hpvcpcC22	hpvcpcC30	hpvcpcC38	hpvcpcC46	hpvcpcC54	hpvcpcC62	hpvcpcC70	hpvcpcC78	hpvcpcC86
RLU	1371	97	137	145	109	97	97	115	103	251	147	151
Ratio		0 22	0 32	0 34	0 25	0 22	0 22	0 27	0 24	0 59	0 34	35
Result	<outlier rejected (Pos2)>											
H												
ID	PC-B	hpvcpcC7	hpvcpcC15	hpvcpcC23	hpvcpcC31	hpvcpcC39	hpvcpcC47	hpvcpcC55	hpvcpcC63	hpvcpcC71	hpvcpcC79	hpvcpcC87
RLU	435	97	131	107	107	109	145	103	109	109	131	591
Ratio		0 22	0 29	0 25	0 25	0 25	0 35	0 24	0 25	0 25	0 30	139
Result												HPV +

A 23: Assay validation of DNA detection of N. gonorrhoea Probe

Assay:	Digene GC-ID	Type:	Single	Area:	A1..B9

Assay is valid

Operator:	ashrafunnessa	Kit Lot #	1929kx01	Exp. Date:	2/18/02	Room Temp:	25

Comment: study

Software rev: 1.1.3

Replicates:	Negative	Positive	QC
Blanks 0	Cal: 3	Cal: 3	Ctrls: 1
Specimens 1			

Number of QC Control types required for assay validation: 0

Assay validation: **Discard Outliers:** **Yes**

Max. NC 500	*Min. NC:* 10	*Max. %CV:* 25	
Max. PC N/A	*Min. PC:* N/A	*Max. %CV:* 25	
Control Ratio PC/NC	*Max:* 20	*Min:* 2	
Mean NC = 217.00	%CV = 3.91	**%CV - valid**	**Mean NC valid**
Mean PC = 938.33	%CV = 2.27	**%CV - valid**	**Mean PC N/A**
Ratio = 4.32	**Ratio: valid**		

Cutoff Calculation:

Cutoff Factor 1 *Cutoff* = Mean PC * Cutoff Factor

*Negative Cutoff = Cutoff * Negative Factor (NF)* *NF* = 1

*Positive Cutoff = Cutoff * Positive Factor (PF)* *PF* = 1

RLU values: **Pos. Cutoff ≥** 938.33 **Neg. Cutoff <** 938.33 938.33 **≤ Equivocal <** 938.33

QC control ratios:

QC control	Max. Ratio	Min. Ratio	Max. %CV	%CV	Ratio	Valid %CV	Valid Ratio

Supervisor's Name: _____ **Date:** _____

A 24: DNA detection report of N. gonorrhoea from Control and CIN specimen

Assay	Area	Operator	Kit Lot #	Valid?	Digene Approved?	Positive Cutoff	Negative Cutoff
Digene GC-ID	A1..B9	ashrafunnessa	1929kx01	Yes	Yes	938.33	938.33

	1	2	3	4	5	6	7	8	9	10	11	12
A												
ID	NC	gcC27	gcC59	gcC67	gcC75	gcC83	gcD9	gcD33	gcD41			
RLU	223	337	201	477	337	343	637	413	259			
Ratio		0.35	0.21	0.50	0.35	0.36	0.67	0.44	0.27			
Result	-	--	--	--	--	--	--	--				
B												
ID	NC	gcC45	gcC60	gcC68	gcC76	gcC84	gcD19	gcD34	gcD42			
RLU	214	825	163	281	511	885	333	95*	341			
Ratio		0.87	0.17	0.29	0.53	0.94	0.35	1.03	0.36			
Result		--	--	--	--	--	--	GC +	--			
C												
ID	NC	gcC49	gcC61	gcC69	gcC77	gcC85	gcD25	gcD35				
RLU	397	1281	183	309	399	285	485	255				
Ratio		1.36	0.19	0.32	0.39	0.30	0.51	0.27				
Result	<outlier rejected>	GC +	--	--	--	--	--	--				
D												
ID	PC-GC	gcC50	gcC62	gcC70	gcC78	gcC86	gcD27	gcD36				
RLU	925	201	305	455	577	663	1085	1597				
Ratio		0.21	0.32	0.48	0.61	0.70	1.16	1.70				
Result		--	--	--	--	--	GC +	GC +				
E												
ID	PC-GC	gcC53	gcC63	gcC71	gcC79	gcC87	gcD28	gcD37				
RLU	963	225	255	385	1025	679	1267	305				
Ratio		0.23	0.27	0.41	1.09	0.72	1.35	0.32				
Result		--	--	--	GC +	--	GC +	--				
F												
ID	PC-GC	gcC55	gcC64	gcC72	gcC80	gcD2	gcD30	gcD38				
RLU	927	169	291	315	513	599	2769	301				
Ratio		0.18	0.31	0.33	0.54	0.63	2.96	0.32				
Result		--	--	--	--	--	GC +	--				
G												
ID	gcC8	gcC57	gcC65	gcC73	gcC81	gcD6	gcD31	gcD39				
RLU	1519	221	543	357	469	993	589	58*				
Ratio	1.72	0.23	0.57	0.38	0.49	1.05	0.60	1.53				
Result	GC +	--	--	--	--	GC +	--	--				
H												
ID	gcC19	gcC58	gcC66	gcC74	gcC82	gcD:	gcD32	gcD40				
RLU	359*	205	161	157	196	487	257	27*				
Ratio	3.62	0.21	0.17	0.16	0.21	0.51	0.27	0.26				
Result	GC +	--	--	--	--	--	--	--				