University of Alberta

Prevention of cervical cancer through the characterization of E6 and E7 mRNA transcriptional activity as biological markers of human papillomavirus infections

by

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ABSTRACT

The quantification of human papillomavirus (HPV) oncogene transcripts, E6 and E7, may be predictive of viral oncogenesis and cancer progression. The main objectives of this study were to determine the HPV genotype prevalence and distribution in Edmonton, Alberta, and characterize a quantifiable association of HPV E6/E7 mRNA expression with the presence of cervical disease. Successful clinical trial design and patient enrolment lead to the first controlled characterization of HPV genotype epidemiology in Alberta. HPV-16 was identified as the most prevalent genotype, followed by several non-vaccine genotypes (HPV-31, -52). Despite rigorous experimentation and a significant correlation between high-risk HPV infection and cervical lesions ($p<0.05$), absolute quantification of viral oncogenesis was unsuccessful. The ability to quantify oncogene transcriptional activity may, in time, revolutionize cervical cancer screening programmes, akin to the Pap smear several decades ago. However, as experienced in this study and in others, great challenges and even greater questions remain unanswered.

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME/DESCRIPTION
Pap	Papanicolaou smear
HPV	human papillomavirus
bps	base pairs
mRNA	messenger ribonucleic acid
NIL	negative for intraepithelial lesion or malignancy
ASCUS	atypical squamous cells of undetermined significance
ASCH	atypical squamous cells of undetermined significance cannot exclude high-grade squamous intraepithelial lesion
AGC	atypical glandular cells
LSIL	low-grade squamous intraepithelial lesion
HSIL	high-grade squamous intraepithelial lesion
SCC	squamous cell carcinoma
AIS	endocervical adenocarcinoma *in situ*
LBC	liquid based cytology
ACCSP	Alberta Cervical Cancer Screening Program
PCR	polymerase chain reaction
CIN	cervical intraepithelial neoplasia
LA	linear array
LR	low-risk HPV type
UR	undetermined-risk HPV type
HR	high-risk HPV type
LEEP	loop electrosurgical excision procedure
CaSki	positive HPV control
C33-A	negative HPV control
cRNA	complementary ribonucleic acid
DNase	Deoxyribonuclease
C_t	threshold cycle
ΔRn	fluorescent emission in a sample normalized to background fluorescence and fluorescence produced in the absence of template
R^2	a measure of the closeness of each data point to the standard curve
no RT	no reverse transcriptase control
ΔC_t	the difference in C_t values produced by amplification in the sample and it's no RT control
MFI	median fluorescent intensities
FITC	fluorescein isothiocyanate
FISH	fluorescent *in situ* hybridization

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Worldwide, cervical cancer is the second most common cancer in women, succeeding breast cancer (1). In 2002, there were an estimated 490 000 new cases, and 270 000 deaths associated with cervical cancer worldwide, with the majority of these new cases occurring in developing countries (1). In Canada, there were an estimated 1900 new cases and 390 deaths attributable to cervical cancer in 2009 (2). The disparity in the incidence and mortality of cervical cancer worldwide is most likely due to the implementation of successful cervical cancer screening programs, which can detect and treat pre-cancers (1). Typically, these programs involve a Papanicolaou (Pap) smear, to examine the exfoliated cells of the cervix, but most recently advancements have been made in the molecular diagnostics of this cervical disease. Research has shown that cancer of the cervix is unique from many other cancers because 99.8% of the cases are linked to a sexually transmitted human papillomavirus (HPV) infection (3). New molecular technologies are thus targeting this infection to identify diagnostic and prognostic markers that lead to the development of cervical cancer.

1.1 HPV EPIDEMIOLOGY

1.1.1 Risk Types

HPV belongs to the family *Papillomaviridae*, with those specifically infecting the genital tract separated into the genus *Alphapapillomavirus* (4). The genus of *Alphapapillomavirus* is subdivided into risk groups; low-risk (LR), undetermined-risk (UR) and high-risk (HR), based on their potential for malignant transformation. Low-risk HPV types, like 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 are commonly found in benign skin lesions like anogenital warts, while high-risk HPV types, like 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are frequently found in precancerous and cancerous malignancies. Although the majority of sexually

active males and females will acquire an HPV infection at least once in their lifetime, the prevalence and distribution of HPV infections varies considerably among different age groups and different stages of cervical disease (1, 2, 5).

1.1.2 Age-Specific Prevalence

The prevalence of HPV infection tends to be highest in women younger than 30 years of age (6). Following this peak, some studies show a steady decline in HPV prevalence with increasing age, while others show a second peak of increasing prevalence around age 45 (6). The disparity in HPV type prevalence in age-specific groups is thought to be due to acquired immunity and physiological changes of the cervix over time (7). Throughout Canada, large genotype prevalence studies have been completed in Ontario (n=955) and British Columbia (n=5000); they both found the highest prevalence of HPV in women who are approximately 20 years of age, with consecutively decreasing prevalence with increasing age (8, 9). A study on Inuit women in Nunavik, Quebec (n=554) produced a U-shaped curve with HPV prevalence at its peak in women 15-19 years of age and then again at 60-69 years of age (10). No data have been published regarding the state of HPV genotype prevalence in Alberta.

1.1.3 Disease-Specific Prevalence

HPV prevalence also varies depending on the severity of cervical disease. Research has shown that as cervical dysplasia becomes more severe there are higher proportions of high-risk HPV types present (11). A worldwide prevalence and genotype distribution study (n=157879) of normal cervical samples found 10.4% of the specimens to be HPV positive and the most common genotypes found were HPV-16, followed by HPV-18, HPV-31, HPV-58 and HPV-52, all of which are high-risk types (12). A study from Italy of a sample population of women with varying degrees of cervical disease (n=626) found that as cervical disease became more severe the proportion of cases infected with any HPV type or a high-risk HPV type increases (13). Not only did the prevalence of high-risk HPV types increase but the distribution of types within an infected

individual included more high-risk types at the sacrifice of low-risk types, whose prevalence decreased, as cervical disease progressed (13, 14). Similar trends have been identified in both Canadian and international studies (9, 11, 15-18).

1.2 HPV CHARACTERISTICS

1.2.1 Genome Organization

HPV is a circular, double-stranded deoxyribonucleic acid (DNA) virus (Figure 1.1). Its genome contains 7900 base pairs and includes 8 genes (E1, E2, E4, E5, E6, E7, L1, and L2) and one non-coding region (long control region). The coding regions are subdivided into late (L) and early (E) regions according to their time of expression during the virus life cycle (19, 20). Their expression is temporally regulated, within the host cell nucleus, through transcription initiation at different promoters appropriately termed the early (P_{97}) and late (P_{742}) promoters (19). The 8 open reading frames (ORFs) in HPV are transcribed as polycistronic messenger RNA (mRNA) that is used for the translation of viral proteins (19).

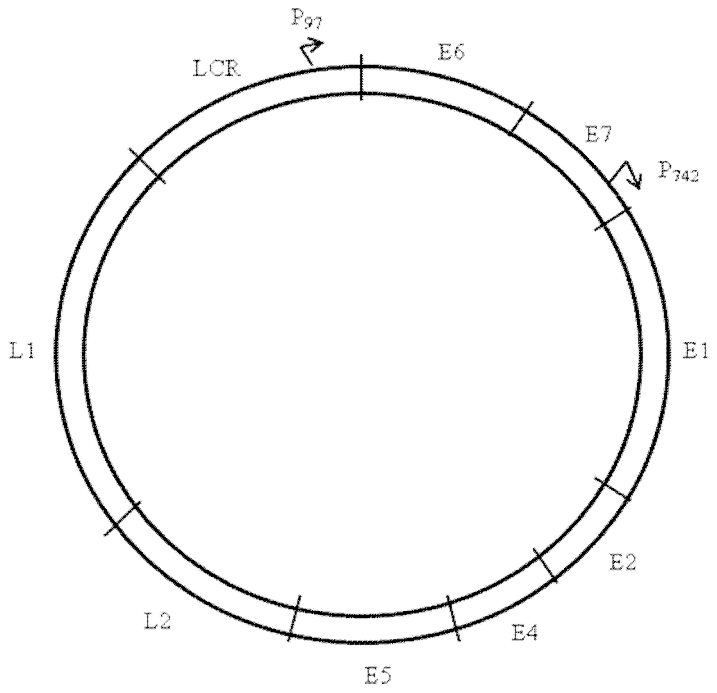

Figure 1.1: Circular organization of the HPV genome, including coding and non-coding regions, and promoters (3, 20).

1.2.2 Viral Genes

The long control region (LCR) or upstream regulatory region (URR) is a sequence required for the regulation of viral replication and transcription (21). It contains the early promoter and binding sites for viral proteins, E1 and E2, and host proteins responsible for HPV expression (20). The E1 and E2 genes encode viral replication proteins (20). The E1 protein functions in the nucleus of the host cell as a helicase and as a recruiter of replication machinery to stabilize the replication fork (22, 23). The E2 protein is active during both replication and transcription; it regulates the activity of the early promoter, stimulates transcription during differentiation, and plays a role in episomal maintenance (23-25). E4 is translated as a fusion protein with E1 and is expressed throughout the virus life cycle (26, 27). It has potential roles in

viral egress, genome amplification and the inhibition of mitosis (28-31). The E5 gene encodes a membrane localised protein that affects host antigen presentation by altering endosome acidification and trafficking (32-34). E5 is expressed throughout the virus life cycle, and typically in higher amounts in cells that have begun differentiation (35). The L1 and L2 genes are expressed late in the virus life cycle from the late promoter and encode the major and minor capsid proteins required for virus assembly. L2 proteins also play a role in the nuclear translocation and recruitment of viral genomes for encapsidation (20).

1.2.3 Viral Oncogenes

E6 and E7 are viral oncoproteins that play a critical role in cervical cancer development, and consequently make good targets for pre-cancer and cancer diagnosis, prognosis, and treatment. Figure 1.2 outlines some of the interactions and effects that occur within the host cell in response to E6 expression that lead to abrogation of the host cell controls. The E6 gene encodes a protein that functions in both the nucleus and cytoplasm (36, 37). It has the ability to bind and degrade the tumor suppressor protein p53, bind PSD-95/discs large/ZO-1(PDZ) domains, and act as a telomerase (38-41). The degradation of p53 is accomplished through interactions with E6 and the E6-associated protein (AP), which leads to the ubiquination of lysines on p53 targeting it for proteolysis (21). The binding of E6 to PDZ domains leads to a similar ubiquination and degradation process (21). Its activity as a telomerase leads to the replication of the telomeres at the ends of chromosomes (21). Overall, these interactions lead to continued progression of the cell cycle, viral replication, DNA synthesis, hyperplasia and immortalization of the infected cells (20) (Figure 1.2).

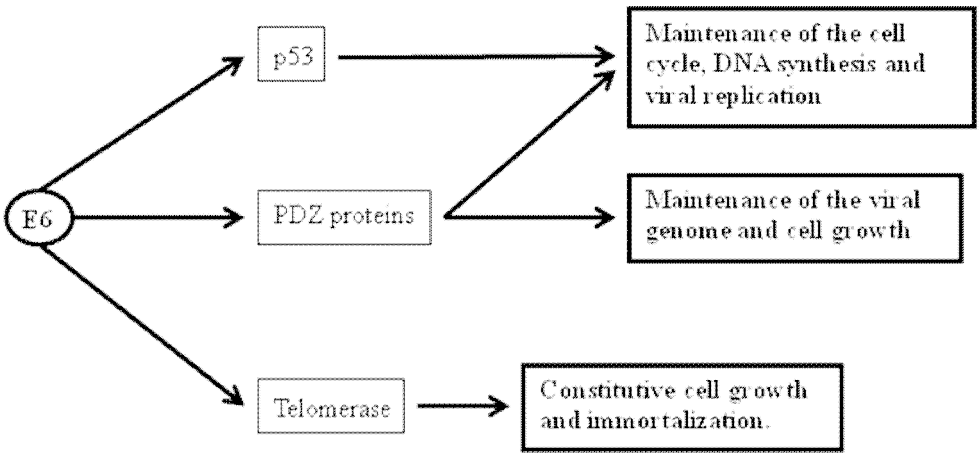

Figure 1.2: The effects of interactions between the HPV E6 gene product and host cell proteins within the host cell (20, 21, 38-41).

The E7 gene encodes a protein that has the ability to bind and degrade tumor suppressor protein pRb, and interact with class I histone deacetylases (HDACs) and cyclin kinase complexes (42-46). Figure 1.3 outlines some of the interactions and effects that occur within the host cell in response to E7 expression. The degradation of pRb occurs through an ubiquitin-mediated process similar to E6 associated degradation (19). HDACs normally act as transcriptional repressors but in the presence of E7, transcription levels remain elevated and the cell cycle continues to progress through S phase (47). E7 stimulates cyclin kinase complexes to phosphorylate host proteins, like pRb, to maintain transcriptional activity of the infected cell (20). The cumulative effect of these interactions with E7 leads to the progression and maintenance of the cell cycle and transcription (20). E7 further contributes to malignant transformation through the induction of genomic instability by influencing centrosome function (48). The cumulative effects of E6 and E7 lead to the malignant transformation, and the immortalization of infected cells. Individually both E6 and E7 have the ability to immortalize and transform host cells however their effects are further amplified when working in synergy (21).

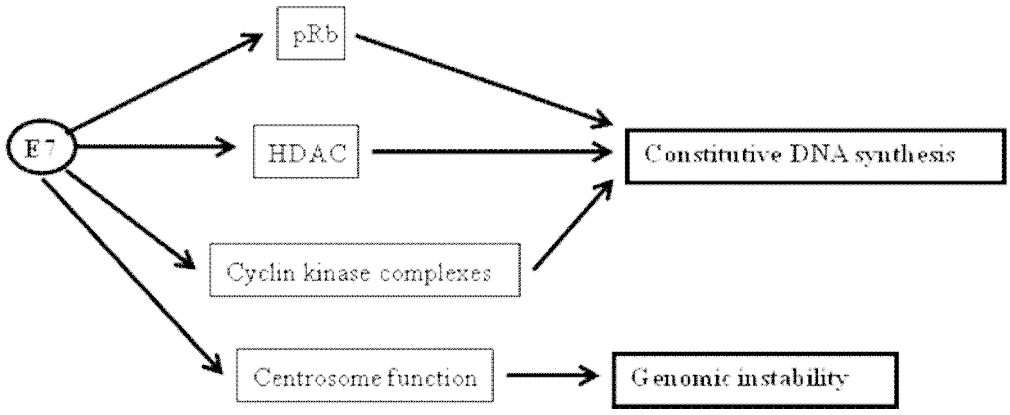

Figure 1.3: The effects of interactions between the HPV E7 gene product and host cell proteins within the host cell (20, 42-48).

1.3 HPV PATHOGENESIS

1.3.1 Site of Infection

In the cervix, HPV infects a group of cells known as the squamo-columnar junction, or the transformation zone (4) (Figure 1.4). The transformation zone is a junction of two cell types, specifically stratified squamous epithelial cells, which line the vagina and the outside of the cervix, and columnar cells, which line the cervical canal. Normally, columnar cells constantly transform into squamous cells by a process known as metaplasia. However, dysplasia refers to the abnormal growth that occurs in this region in response to an HPV infection.

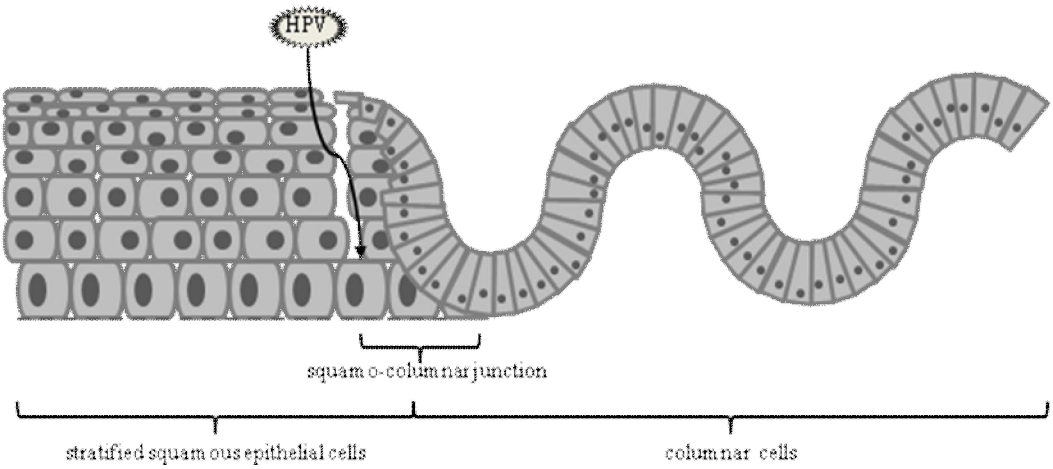

Figure 1.4: A representation of the squamo-columnar junction where HPV infections occur within the cervix. The gap between the cells in the junction is representative of a microabrasion which allows access to HPV to the basal layer of the epithelium.

1.3.2 Viral Life Cycle

HPV gains access to the basal layer of the epithelium through microabrasions, in order for the infection to establish it must reach cells in the stratified squamous epithelium that are capable of differentiation and replication (3) (Figure 1.4). Once inside a host cell in the basal layer, the life cycle of HPV becomes highly coordinated with the processes occurring within that cell. Cells from the basal layer begin to divide, migrate towards the apical surface, differentiate and accumulate keratin. Normally, once these host cells reach the surface, cell division and DNA synthesis has stopped and the cells are no longer actively functioning. In the presence of HPV and viral oncoproteins E6 and E7, the cells continue to divide and synthesize DNA in order to maintain viral replication, transcription and translation by manipulating host cellular machinery. Once HPV-infected cells have reached the surface, assembled infectious virus particles are released from the cell and can establish additional infections elsewhere using the same mechanisms as indicated above.

1.3.3 Infectivity of Risk Groups

High-risk and low-risk HPV types differ in their ability to infect and transform host cells. This difference is most likely due to differences in the expression, activity and binding affinity of oncoproteins E6 and E7. There are a number of events that occur during a high-risk HPV infection that ensure the continuation of cellular processes to maintain viral replication. Infections with a high-risk HPV type typically lead to the integration of the viral genome into the host cell DNA. This integration disrupts the E2 gene, which encodes a protein that regulates HPV expression, leading to the constitutive expression of E6 and E7 by host cell promoters now continually activated by viral factors abrogating normal host cell controls (49). In comparison, low-risk HPV type genomes are maintained as episomes outside of the host cell DNA and expression of the viral genes is regulated normally (50). The activity of the E2 protein is distinctly different among the risk groups. Its functions as a transcriptional activator are considerably decreased in low-risk HPV types because affinity with host transcription factors and co-activators is much weaker (25). The translation efficiency of E6 and E7 is stabilized in high-risk HPV types because of alternative splicing mechanisms occurring post-transcriptionally. The efficiency of translation is increased because the splicing that occurs increases the distance between the end of first ORF and the beginning of the second ORF allowing for translation termination reinitiation to occur with ease (51). No alternative splicing mechanisms have been identified in low-risk HPV types therefore translation of the oncogenes is much less effective (52). The cumulative effects produced in high-risk HPV type infections leads to more efficient transcription and translation of E6 and E7, which contributes to their ability to successfully immortalize and transform host cells.

1.4 CERVICAL CANCER AND HPV DETECTION METHODS

1.4.1 Current Cervical Cancer Screening Methodologies

The Canadian Cancer Society indicates that both the incidence and mortality of cervical cancer has been significantly decreasing across Canada (2). These decreases are attributed to the

implementation of cervical cancer screening programmes across the country involving a Pap smear (2). The Pap smear is a diagnostic tool whereby exfoliated cervical cells are sampled from the transformation zone, stained, fixed and visualized microscopically for abnormalities (53). Cytological abnormalities can be identified and then classified according to the 2001 Bethesda classification system as NIL (negative for intraepithelial lesion or malignancy), ASCUS (atypical squamous cells of undetermined significance), ASCH (atypical squamous cells of undetermined significance cannot exclude high-grade squamous intraepithelial lesion), AGC (atypical glandular cells), LSIL (low-grade squamous intraepithelial lesion), HSIL (high-grade squamous intraepithelial lesion), SCC (squamous cell carcinoma), AIS (endocervical adenocarcinoma *in situ*), or adenocarcinoma (54). In screening programmes, most significant abnormalities identified on a Pap smear are considered precursors to the development of many cancers of the cervix. Women with abnormal cytology are considered to be at a higher risk of cervical cancer development and are monitored differently than those with normal cytology.

1.4.2 The Pap Smear

A meta-analysis of 62 studies revealed that the Pap smear alone has a mean sensitivity of 58% and a mean specificity of 69% (53). Limitations of the previous study, however, indicate that different abnormal cytology endpoints were used for the calculation of sensitivity among the 62 studies included in the analysis; standardization of this end point would allow for better comparisons of this measure (53). In general, sensitivity is defined as the proportion of women with a cervical precursor lesion that are correctly identified as such, while specificity is the proportion of women without a cervical precursor lesion that are correctly identified as such. Sensitivity and specificity are calculated in comparison to results obtained from colposcopy, the gold-standard, which involves magnification and visualization of the cervix to identify and potentially biopsy areas of abnormality (55). The majority of the inaccuracies associated with Pap smear are due to false negative results, limited sensitivity, caused by specimen inadequacies, sampling, technical and interpretation errors (56, 57). On a negative smear, specimen

inadequacies are outlined as an under-representation of cellularity and/or cells included in the transformation zone (i.e. endocervical component), or the presence of obscuring blood, mucus, or inflammatory cells. Sampling and technical errors include inappropriate sample collections and sample preparations containing an inadequate number of cells, obstructing debris, blood or mucus on the smear (53, 58, 59). Interpretation errors are due to the subjective and repetitive nature of interpreting the slides, which is then further confounded by the individual cytotechnologists training, competence, and motivation (60).

1.4.3 Advancements

Technologies have been developed to combat these errors and inadequacies currently challenging the success of the conventional Pap smear. Liquid based cytology (LBC) preservatives, like SurePath® (Becton Dickinson Diagnostics) and PreservCyt® (Hologic™), allow better transfer of cellular material from the collecting device to the slide, while also allowing for slide preparation and primary visualization to be automated (61). These liquid based preservatives are typically a solution containing a blend of ethanol or methanol with water, and they serve as good media to preserve the integrity of both viral DNA and RNA so that residual sample can be used for molecular testing (62). ThinPrep® (Hologic™) technology works in conjunction with the liquid based preservatives to allow for the automated preparation of slides to create a uniform cell monolayer free of debris, while it also contains an automated system for the primary screening of slides (60, 63). The ThinPrep® Imaging System will help to reduce some of the interpretation errors that occur with conventional smears. In combination, the use of both ThinPrep® automated slide preparation and visualization has lead to a decrease in the number of inadequate samples prepared, while also potentially increasing Pap smear sensitivity through the visualization of higher quality smears. Ultimately, the use of liquid based preservatives in conjunction with ThinPrep® technology allows for the increased detection of cellular abnormalities further increasing the sensitivity of the Pap smear, and allowing for molecular testing to take place with ease.

1.4.4 Limitations

Although the incidence and mortality of cervical cancer has been steadily decreasing, current Pap smear technologies lack the ability to distinguish between women with persistent, clinically significant cytological abnormalities and HPV infections from those with transient, regressive cytological abnormalities and HPV infections. The presence of the virus at a given time point does represent an established infection, but further confounding its presence is its ability to regress in the majority of patient cases. Recent diagnostic tests and kits have been developed to be used in conjunction with the Pap smear to attempt to discriminate between these cases. Current Alberta Cervical Cancer Screening Program (ACCSP) Guidelines recommend increased screening of all women with ASCUS and LSIL, including Pap smears and HPV testing where available and appropriate, and referral for colposcopy of all women with persistent ASCUS/LSIL, ASCH, HSIL, AGC, AIS or SCC (63).

1.5 HPV DETECTION AND GENOTYPING

1.5.1 DNA-Based Assays

Used as a secondary identification tool for abnormal Pap smears, HPV molecular tests have the ability to identify HPV infections and suggest etiology of indeterminate cytological abnormalities. Numerous molecular tests have been developed to identify the presence of HPV in cervical smears. Below are two examples of HPV molecular tests, made by two different companies, that both use HPV DNA as their target to identify HPV infections. Hybrid Capture® II high-risk HPV DNA (Digene) test is an *in vitro* nucleic acid hybridization assay that allows for the detection of DNA from 13 different high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). This technology involves the hybridization of target HPV DNA to RNA probes. These hybrids are then immobilized, reacted with alkaline phosphatase, and detected by chemiluminesence that is measured by a luminometer. Weaknesses of this test include its qualitative nature, its inability to genotype the infecting HPVs and to identify multiple infections.

HPV genotyping assays allow for the identification of multiple infections and over time can also identify type-specific persistence in women who are at a higher risk of developing cervical disease (64). The Linear Array HPV Detection and Genotyping Kit (Roche) allows for both the detection and genotyping of 37 different HPV types, including low-risk (6, 11, 40, 42, 54, 61, 64, 70, 71, 72, 81, CP6108), undetermined-risk (55, 62, 66, 67, 83, 84), and high-risk types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 69, 73, 82, IS39). This assay involves the amplification of the L1 HPV DNA sequence with biotin-labelled primers. Target amplicons are then detected by binding with immobilized, complementary oligonucleotide probes that react with streptavidin horseradish peroxidase to oxidize 3, 3', 5, 5' tetramethylbenzidine. Weaknesses of this test include its qualitative nature, however this test can discriminate HPV genotypes and multiple infections. The main disadvantage of both of these tests is the use of HPV DNA as a predictor of HPV infections. As stated previously, the majority of women will be infected with HPV in their lifetime and very few will develop cervical cancer; this lends to the argument that the detection of HPV DNA does not correlate well with the development of cervical disease (65).

1.5.2 RNA-Based Assays

The detection of HPV RNA, specifically the transcripts of viral oncogenes E6 and E7, may be more predictive of viral oncogenesis and cancer progression (66). The PreTect™ HPV Proofer Assay (Norchip, AS) allows for the detection of E6/E7 mRNA of five high-risk HPV types in cervical samples. This assay is based on real-time multiplex nucleic acid sequence based amplification (NASBA) (67). NASBA is a primer-dependent technology that allows for the amplification of multiple target single stranded RNA molecules under isothermal conditions (68). Amplification in this assay is accomplished through the use of two-primer and molecular beacon probe sets and detection is completed using a fluorescent reader (65). This assay allows for the differentiation, or genotyping, of the HPV types identified and it also allows for the identification of multiple infections. Studies have shown that this assay has a similar sensitivity to polymerase chain reaction (PCR) but its specificity is much higher therefore decreasing the number of false

positive results (69). An important weakness of this assay, similar to the HPV DNA-based molecular tests is its qualitative nature.

1.5.3 HPV RNA Quantification

Wang-Johanning, *et al.* (2002) found that E6 and E7 RNA levels increased as the severity of cytology increased, suggesting a direct link between the amount of transcript and the severity of cervical disease (70). Knowing the quantity of oncogene transcript present in a cervical sample could further increase the accuracy of diagnosis and also be a more appropriate predictor of disease progression.

The quantification of HPV viral mRNA in cervical samples requires knowledge of the collected amount of cervical cells. In the absence of this a sample with very few exfoliated cells from a woman with HSIL cytology, and a sample with a significant number of exfoliated cells from a woman with ASCUS or LSIL could both contain a similar amount of viral transcript, although these samples are very different in terms of clinical significance. De Boer, *et al.* (2007) desired to determine the prognostic value of the HPV DNA copy number and E6/E7 mRNA expression (71). This study used HPV positive cervical cancer biopsies stained with keratin and vimentin to determine the proportion of tumor cells present using flow cytometry. This proportion was then used to normalize the amount of HPV DNA and mRNA present in the samples by differentiating HPV-infected, tumor cells from uninfected, normal cells. Vimentin is used in flow cytometric analysis to differentiate the cells that have undergone malignant transformation and become invasive from those that have not. The use of vimentin limits the prognostic value of this approach because it cannot be applied to, or used to differentiate, non-invasive, precancerous states, which have different features cytometrically than tumor cells. Narimatsu, *et al.* (2005) described the simultaneous immunophenotyping and fluorescent *in situ* hybridization (FISH) for HPV E6 and E7 mRNA (72). Using liquid based cytology samples, antibodies, FITC-labelled oligonucleotide probes and a flow cytometer, they were able to separate the heterogeneous cervical cell populations into individual components and determine the number of cells in each

component expressing E6/E7 mRNA. Further, they were able to show that as cytology becomes more severe, the number of samples expressing E6/E7 mRNA increases accordingly. The detection and quantification of HPV RNA, specifically the transcripts of viral oncogenes E6 and E7, may be more predictive of viral oncogenesis and cancer progression (66). By identifying these persistent, active infections, screening programs would be able to triage women more appropriately and prevent over-treatment and under-screening.

1.6 HYPOTHESIS

The quantification of HPV E6 and E7 oncogene transcripts in cervical infection is a more sensitive and appropriate predictor of cervical disease than current diagnostic markers**.**

1.6.1 Objective

The main objective was to characterize a quantifiable association of HPV E6 and E7 mRNA expression with specific stages of cervical cell dysplasia.

1.6.2 Study Aims

1. Determine the genotypes of HPV present within the patient cervical cell samples.

Baseline specimen characterization of liquid-based cervical samples collected from women referred to the colposcopy clinic was established using a qualitative assay for HPV genotype-specific L1 DNA (Linear Array Detection Kit and HPV Genotyping Kit, Roche). This test validated the presence and genotype of infecting HPVs within the cervical cell samples.

2. Determine the proportion of cells expressing E6/E7 mRNA within the liquid based cervical cell specimens.

Normalization was completed to control for the number of normal and HPV-infected cervical cells within a given specimen at the time of collection. This was accomplished using a previously described method that utilizes flow cytometry to detect HPV-infected cervical cells that are expressing E6/E7 mRNA within the total cell population (72). This approach allowed for the identification of the proportion of cells expressing HPV E6/E7 mRNA within the total cell mass of each specimen.

3. Quantify the level of E6/E7 mRNA present in normalized cervical specimens using real-time reverse transcription PCR.

Cervical cell specimens were extracted for total RNA and reverse transcribed. Absolute quantification of HPV-16 E6/E7 mRNA was then completed via multiplex real-time PCR using an ABI amplification platform and nucleic acid primer pairs and TaqMan® probes. Standard curves were generated with each PCR experiment and absolute quantification was accomplished using a standard RNA dilution series produced from an *in vitro* transcription reaction of a plasmid constructed to contain the target E6/E7 mRNA sequence.

4. Correlate the level of E6/E7 mRNA expression with the specific stages of precancerous cervical dysplasia.

Cytology and histology results were obtained for each cervical sample submitted. Correlations were then drawn between the baseline cytology/histology result and the quantity of HPV-16 E6/E7 mRNA present.

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CHAPTER 2

HPV genotype prevalence and distribution in a colposcopy population, Edmonton, Alberta, Canada

2.1 INTRODUCTION

HPV is a known etiological agent required in the development of cervical cancer (1). Upon the implementation of cervical cancer screening programs, a significant decrease was identified in both the mortality and incidence associated with cervical cancer (2). This decrease is attributable to the incorporation of Pap smear cytology, which can distinguish between normal, precancerous and cancerous cells of the cervix. Although the implementations of screening programs worldwide have produced significant decreases in the incidence of cervical cancer, the sensitivity and specificity of the Pap smear remains suboptimal (3). More recently, this has lead to a plateau in the incidence and mortality rates associated with cervical cancer. HPV genotyping and detection assays have been developed to help circumvent this disparity. One of the key roles of HPV genotyping is the accurate identification of high-risk HPV infections in women and their concomitant risk of developing cervical disease (4-6). These assays also allow the scientific and medical communities to identify the regional HPV genotype prevalence and distribution within different populations of women: screening versus colposcopy populations. A routine screening sample population would provide information regarding the HPV genotype prevalence in a group of women with minor or no cervical abnormalities. A colposcopy based sample population would provide information regarding the distribution and prevalence of high-risk HPV types in a group of women with cervical abnormalities. This sample population could contribute significant knowledge concerning the etiological relationship between cervical cancer development and high-risk HPV infections, and the efficacy of prophylactic vaccines designed to prevent HPV-associated cervical cancer.

The main objective was to characterize the HPV genotype prevalence in a population of women referred for colposcopy in Edmonton, Alberta. HPV genotype prevalence in cervical

disease in any patient population in Alberta has not been investigated to date. Further analysis of the infecting HPV types and cytological/histological diagnoses was completed on a small subset of patients who had submitted follow-up samples.

2.2 MATERIALS AND METHODS

2.2.1 Study Design

Study enrolment targeted women within the routine cervical cancer screening program that had been referred for colposcopic examination. Prior to revision in 2009, the ACCSP guidelines required a minimum of two ASCUS cytology results from screening visits occurring at 6-month intervals within a two year period. Patients meeting this minimum were then referred for colposcopy. Women presenting at these routine visits with more severe cytology (HSIL) were also referred. Once referred, women were followed up based on the outcome of their colposcopy visit. Women presenting with high-grade lesions (CIN2/3) were treated and then follow-up visits were required every 6 months for 2 years. Women with low-grade or no lesions received no treatment (NIL, or CIN 1), but were required to also schedule follow-up visits every 6 months for 2 years. Clinical specimens for this study, including those representing all cytological and histological categories, were collected from the Colposcopy Clinic at the Cross Cancer Institute in Edmonton, Alberta. Patient enrolment required informed consent and samples to be collected at the initial, 6-month and 12-month follow-up visits. Colposcopic examination included a Pap smear for routine cytological analysis, a LBC (PreservCyt®, Hologic, Marlborough, Massachusetts) specimen for HPV genotyping, and a cervical biopsy for histological analysis. Cytological classification was assigned according to the Bethesda 2001 system (NIL, ASCUS, LSIL, ASCH, HSIL, AGC, AIS, or SCC) (7). Histologically, specimens were categorized according to severity of cervical disease as cervical intraepithelial neoplasia (CIN) of grades 1, 2 or 3, with grade 3 being the most severe. Histology results classified as “squamous metaplasia”, “chronic inflammation”, “inflammation”, “reactive changes”, “benign glandular epithelium”, and

"inflamed transformation zone", were graded as No CIN for this study. Histology results classified as "potential SIL", "small foci of SIL ungraded", and "mild squamous atypia", were graded as CIN 1 for this study. Any specimens submitted with missing information (missing cytology reports, histology reports, and/or demographic information) or deemed inadequate through molecular testing were excluded in the analysis. Clinical histories outlining previous and current cytology and histology reports were obtained for all patients included in the study in order to characterize the sample population upon entrance into the study, and also throughout the follow-up period. Upon entrance into the study two distinct sub-populations of women were identified within the colposcopy population: (1) women who have a history of colposcopic examination for low-grade abnormalities and/or high-grade abnormalities that may have received treatment (n=21);or (2) women who do not have a history of colposcopic examination, and are now being referred for the presence of potential precursors to the development of cervical cancers (n=194). HPV genotype prevalence was calculated at the initial study visit, stratified according to colposcopic history and analyzed according to histological diagnosis obtained at the initial study visit. In this analysis, indeterminate specimens were inclusive of those specimens where no biopsy was taken, or they were inadequate for histological analysis and no histological diagnosis was given. Ethics approval for this study was granted by the Health Research Ethics Board at the University of Alberta, and the Alberta Cancer Research Ethics Committee at Alberta Health Services.

2.2.2 Specimen Collection

Specimens were collected and genotyped for the study from July 2008 to December 2009. Exfoliated cervical cells were collected with a spatula/endocervical brush combination prior to acetic acid treatment and/or colposcopic biopsy by a collecting physician. The collecting instruments were rinsed in PreservCyt® as per the manufacturer's protocol and retained in their respective LBC vial following collection, A study has shown that discarding the collection instruments following collection can lead to the loss of up to 37% of the cellular material collected

potentially impeding the adequacy of both Pap and molecular analysis (8). Samples were maintained at room temperature for up to 2 months prior to analysis.

2.2.3 DNA Extraction

Specimens were homogenized through inversion, and then aliquoted and centrifuged for 20 minutes at 13000xg, prior to re-suspension in sterile PBS. The pre-DNA extraction steps followed have been described elsewhere (9). DNA was extracted using the MagNA pure LC 2.0 System (Roche Diagnostics Canada, Laval, Quebec) using the DNA Isolation Kit-I and the "blood cell high performance protocol". In brief, the cells were disrupted and proteins denatured by lysis buffer and proteinase K, respectively. The released nucleic acids were bound to magnetic glass particles, washed and eluted. HPV positive and negative controls were provided by the manufacturer.

2.2.4 PCR Amplification

Specimen characterization was established using a qualitative assay for HPV genotype-specific L1 DNA (Linear Array (LA) Detection Kit and HPV Genotyping Kit, Roche Diagnostics Canada, Laval, Quebec). This test validated the presence of HPV within the cervical cell samples and identified the genotype of the infecting HPV. The LA Genotyping test (Roche Diagnostics Canada, Laval, Quebec) has the capacity to amplify 37 different low-risk (6, 11, 40, 42, 54, 61, 64, 70, 71, 72, 81 and CP6108), undetermined-risk (55, 62, 66, 67, 83, 84), or high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 69, 73, 82 and IS39); details regarding changes in HPV genotype nomenclature have been stated elsewhere (10). A pool of 37 different biotinylated primer pairs directed against a 450-bp region of L1 gene in the HPV genome were used to amplify the infecting HPV type(s), and a primer pair directed against the human β-globin gene was included to ensure specimen adequacy. Sample reactions contained 10 µL of extracted DNA and 40 µL of nuclease-free water while control reactions contained 50 µL of extracted control DNA; each reaction contained 50 µL of master mix, which was provided by the

manufacturer, and the reaction assembly was completed to a final reaction volume of 100 µL. Amplification was performed using a 96-well GeneAmp® PCR System 9700 (Applied Biosystems) with a gold-plated silver sample block using the following cycle parameters: 50 °C for 2 minutes, 95 °C for 9 minutes, 40 cycles of 95 °C for 30 seconds, 55°C for 1 minute, and 72 °C for 1 minute at a ramp rate of 50%, and 72 °C for 5 minutes; the samples were then held indefinitely at 72 °C. Once amplification was complete, the amplicons were denatured using 1.6% sodium hydroxide and stored at 4 °C.

2.2.5 LA Hybridization

HPV genotyping was completed using the LA HPV Detection Kit (Roche Diagnostics Canada, Laval, Quebec). HPV genotyping strips, composed of nylon, contained 37 HPV-type specific bovine serum albumin (BSA)-conjugated oligonucleotide probes as well as probes corresponding to high and low levels of the human β-globin gene which control for specimen adequacy. Hybridization of the denatured amplicon was completed through the addition of 0.2% SSPE and 0.025% SDS (hybridization buffer) warmed to 53±2 °C to each genotyping strip. Denatured amplicon was added and the reaction was incubated at 53±2 °C for 30 minutes, with shaking at 60 rpm in a shaking water bath. A series of stringent wash steps were completed using 0.05% SSPE and 0.005% SDS (wash buffer) at room temperature and at 53±2 °C both with shaking at 60 rpm. Once washed, streptavidin–horseradish peroxidase conjugate was added to each strip. The strips were washed to remove unbound materials, and a solution of hydrogen peroxide and 3, 3', 5, 5' tetramethylbenzidine (TMB) was added. Infecting HPV types were genotyped and β-globin controls were identified on the strip using the LA HPV Genotyping Test Reference Guide. Samples that were HPV positive had a blue precipitate which corresponded to particular HPV genotypes, as well as blue precipitate corresponding to both the high and low β-globin controls. Samples that were HPV negative only had blue precipitate corresponding to the both high and low β-globin controls. Samples that did not have blue precipitate for either of the β-globin control genes were assumed to be inadequate for analysis.

2.2.6 Statistical Analysis

Pair-wise comparisons were completed using 2 x 2 contingency tables and statistical significance was determined using the Fisher's Exact Test (http://www.langsrud.com/fisher.htm).

2.3 RESULTS

2.3.1 Specimen Demographic

We enrolled 220 patients into the study and collected a total of 254 LBC specimens; 218/254 (85.8%) initial study visit samples, 31/254 (12.2%) 6-month follow-up study samples, 3/254 (1.2%) 12 month follow-up study samples, and 2/254 (0.8%) were missing patient information. Of the initial study visit samples, 1 (0.4%) was inadequate for genotyping, and 2 (0.8%) were inadequate for cytological and/or histological analysis; these specimens were excluded from further analysis. Of the initial study visit samples, over 70% of the patients were less than 40 years of age (Figure 2.1); 6.0% (13/215) were $\leq$19 years, 44.2% (95/215) were 20 to 29 years of age, 24.7% (53/215) were 30 to 39 years of age, 13.5% (29/215) were 40 to 49 years of age, 7.4% (16/215) were 50 to 59 years of age, and 4.2% (9/215) were over the age of 60. At the initial study visit, 90.2% (194/215) of the sample population had never had a colposcopic examination (i.e. initial colposcopy visit), while 9.8% (21/215) had a history of colposcopic examination for cervical lesions of varying degrees of severity. At the initial study visit, half of the entire patient population showed no evidence of cytopathology (47.4% of 215, NIL) while another 20% had HSIL (42/215); 22/215 (10.2%) were LSIL, 32/215 (14.9%) were ASCUS, 10/215 (4.7%) were ASCH, 2/215 (0.9%) were AGC NOS, 1/215 (0.5%) were AIS, 1/215 (0.5%) were SCC, and 3/215 (1.4%) had no smear taken (Figure 2.2). In stratifying the patient population based on their colposcopic history, a similar distribution of cytology cases was identified in those women presenting at their initial colposcopy visit, in comparison to the entire patient population (Figure 2.3A), while those with a history of colposcopy showed a significantly higher proportion of LSIL ($p<0.05$) (Figure 2.3B). Histologically, at the initial study visit, 77/215

(35.8%) had no CIN, 33/215 (15.3%) were CIN 1, 78/215 (36.3%) were CIN 2/CIN 3, and 27/215 (12.6%) were indeterminate (Figure 2.4). In stratifying the patient population based on their colposcopic history, a similar distribution of histology cases was identified in those women presenting at their initial colposcopy visit, in comparison to the entire patient population (Figure 2.5A). No significant differences were identified in the histological distribution among the entire patient population and those with a history of colposcopic examination (Figure 2.5B). Specimens categorized as CIN 2 or CIN 3 were combined for analysis. Indeterminate specimens had no biopsy results because no biopsy was taken, or the specimen was inadequate for histological analysis.

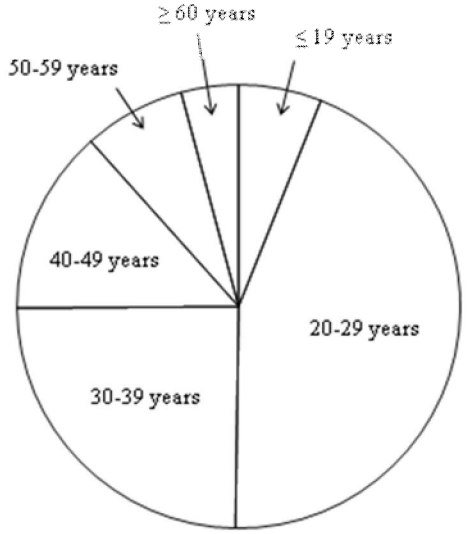

Figure 2.1: Age distribution of the sample population (n=215) at the initial study visit.

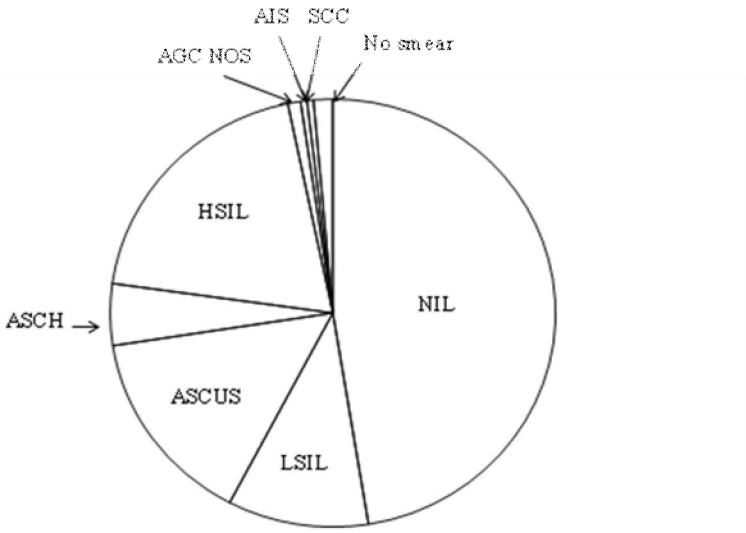

Figure 2.2: Cytology distribution of the sample population (n=215) at the initial study visit.

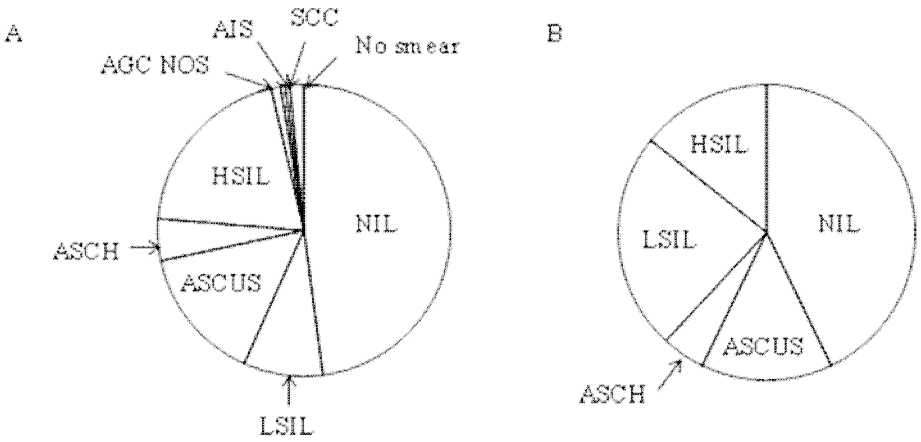

Figure 2.3: Stratification of the cytology distribution of the sample population at the initial study visit based on history of colposcopic examination. (A) Cytology distribution in women with no history of colposcopic examination (n=194). (B) Cytology distribution in women with a history of colposcopic examination (n=21).

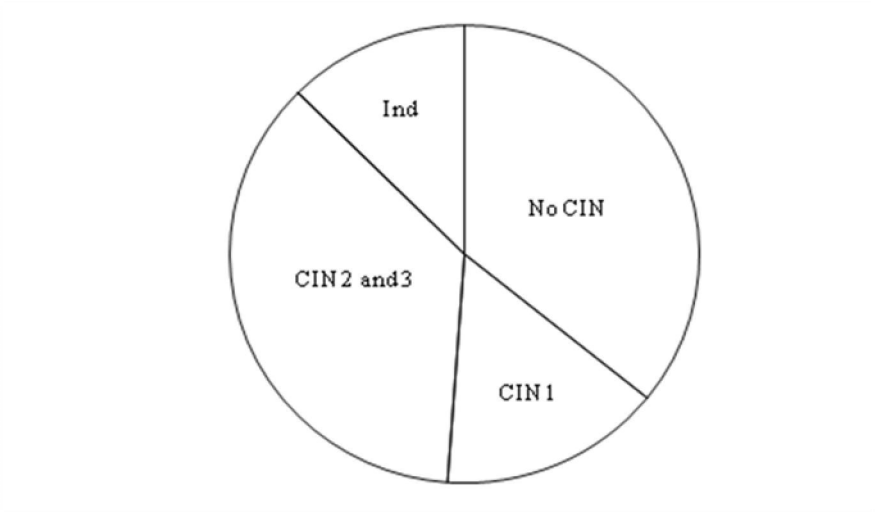

Figure 2.4: Histology distribution of the sample population (n=215) at the initial study visit.

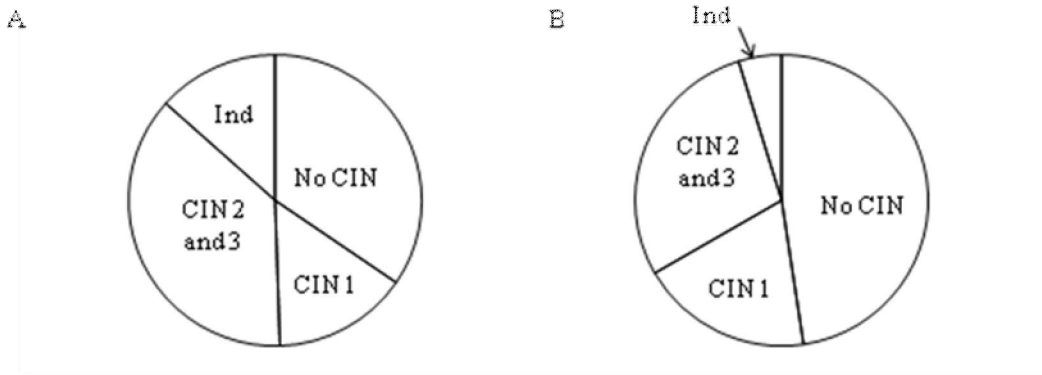

Figure 2.5: Stratification of the histology distribution of the sample population at the initial study visit based on history of colposcopic examination. (A) Histology distribution in women with no history of colposcopic examination (n=194). (B) Histology distribution in women with a history of colposcopic examination (n=21).

2.3.2 HPV Prevalence and Distribution

Overall, 80.0% (172/215) of the study population was HPV positive and 32 different genotypes were identified. Patients infected with multiple HPV genotypes represented 43.3% (93/215) of the population, with 95.7% (89/93) of these harbouring one or more high-risk genotypes. Of the patients infected with a single HPV type (36.7%), 70/79 (88.6%) contained a high-risk HPV type. The 6 most prevalent HPV genotypes identified were 16 (36.7%), 31 (13.0%), 52 (9.3%), 39, 42, and 56 (7.9%), all of which are high-risk genotypes except for HPV-42 (Table 2.1).

The most prevalent HPV genotypes were also identified within the group of women presenting for their first colposcopy visit, in comparison to those with a history of colposcopic examination at their initial study visits (Table 2.2). The overall percentages of HPV-positive samples, single infections, and multiple infections within each group were not significantly different. Also, no significant differences were identified in the percentage of high-risk, undetermined-risk, or low-risk HPV type infections among the groups. With regards to HR-HPV type infections, women lacking colposcopic history at the initial study visit had a higher prevalence of HPV-16 infections ($p<0.05$), while those presenting with a history of colposcopic examination had a higher prevalence of HPV-73 infections ($p<0.05$). The 6 most prevalent types of HPV within women at their initial colposcopy and study visits were HPV-16 (39.2%) followed by HPV-31 (11.9%), HPV-52 (9.8%), HPV-56 (8.8%), HPV-42 (8.2%), and HPV-18 (7.2%). The most prevalent types in women with a history of colposcopy were HPV-31 (23.8%) followed by HPV-16, -39 and -73 (16.3%), and HPV-53 and -84 (9.5%).

Table 2.1: Prevalence and distribution of HPV infections in specimens collected from women referred for colposcopy with abnormal cytology results [number of specimens (%)].

HPV Status		No CIN	CIN 1	CIN 2 and CIN 3**	Ind***	Total
		n = 77	n = 33	n = 78	n = 27	n = 215
Positive*		50 (64.9)	27 (81.8)	75 (96.1)	20 (74.1)	172 (80.0)
Single		33 (42.9)	7 (21.2)	31 (39.7)	8 (29.6)	79 (36.7)
Multiple		17 (22.1)	20 (60.6)	44 (56.4)	12 (44.4)	93 (43.2)
Multiple with HR		17 (22.1)	18 (54.5)	43 (55.1)	11 (40.7)	89 (41.4)
HR Types	16	18 (23.4)	5 (15.1)	49 (62.8)□	7 (25.9)	79 (36.7)
	18	6 (7.8)	1 (3.0)	7 (9.0)□	1 (3.7)	15 (7.0)
	31	6 (7.8)	5 (15.1)	17 (21.8)	0	28 (13.0)
	33	1 (1.3)	0	4 (5.1)	0	5 (2.3)
	35	2 (2.6)	2 (6.0)	3 (3.8)	0	7 (3.2)
	39	4 (5.2)	6 (18.2)	6 (7.7)	1 (3.7)	17 (7.9)
	45	1 (1.3)	1 (3.0)	0	2 (7.4)	4 (1.9)
	51	3 (3.9)	2 (6.0)	6 (7.7)	0	11 (5.1)
	52	3 (3.9)	3 (9.1)	13 (16.7)□	1 (3.7)	20 (9.3)
	53	3 (3.9)	1 (3.0)	7 (9.0)	0	11 (5.1)
	56	1 (1.3)	7 (21.2)	3 (3.8)	6 (22.2)	17 (7.9)
	58	1 (1.3)	3 (9.1)	5 (6.4)	2 (7.4)	11 (5.1)
	59	0	3 (9.1)	1 (1.3)	0	4 (1.9)
	68	0	2 (6.0)	0	1 (3.7)	3 (1.4)
	73	5 (6.5)	2 (6.0)	2 (2.6)	0	9 (4.2)
	82	1 (1.3)	0	1 (1.3)□	0	2 (0.9)
Any HR		43 (55.8)	25 (75.8)	73 (93.6)	17 (63.0)	158 (73.5)
LR Types	6	1 (1.3)	4 (12.1)	2 (2.6)	1 (3.7)	8 (3.7)
	11	1 (1.3)	0	0	1 (3.7)	2 (0.9)
	40	1 (1.3)	1 (3.0)	0	1 (3.7)	3 (1.4)
	42	5 (6.5)	3 (9.1)	5 (6.4)	4 (14.8)	17 (7.9)
	54	3 (3.9)	0	8 (10.2)	1 (3.7)	12 (5.6)
	61	2 (2.6)	0	1 (1.3)	2 (7.4)	5 (2.3)
	70	0	2 (6.0)	4 (5.1)	1 (3.7)	7 (3.2)
	72	1 (1.3)	1 (3.0)	0	0	2 (0.9)
	81	1 (1.3)	3 (9.1)	1 (1.3)	0	5 (2.3)
	CP6108	4 (5.2)	3 (9.1)	3 (3.8)	2 (7.4)	12 (5.6)
Any LR		11 (14.3)	12 (36.4)	20 (25.6)	10 (37.0)	53 (24.7)
UR Types	55	3 (3.9)	2 (6.0)	3 (3.8)	0	8 (3.7)
	62	3 (3.9)	3 (9.1)	3 (3.8)	3 (11.1)	12 (5.6)
	66	2 (2.6)	1 (3.0)	5 (6.4)	3 (11.1)	11 (5.1)
	67	1 (1.3)	2 (6.0)	3 (3.8)	1 (3.7)	7 (3.2)
	83	1 (1.3)	2 (6.0)	0	0	3 (1.4)
	84	4 (5.2)	5 (15.1)	2 (2.6)	0	11 (5.1)
Any UR						

* HPV positivity (%) was determined by the division of the number of HPV positive specimens by the total number of specimens within a given category (i.e. No CIN HPV positivity (%) = 50/77 = 0.649)

** Includes 3 samples with adenocarcinoma *in situ* (□) and 2 samples with vaginal intraepithelial neoplasia

*** Specimens were deemed indeterminate because no biopsy was taken or they were inadequate for histological analysis

Table 2.2: HPV genotype prevalence and type distribution among the sample population stratified based on history of colposcopic examination.

HPV Status		No History*	Colposcopic History
		n=194	n=21
Positive		155 (79.9)	17 (80.9)
Single		70 (36.1)	9 (42.9)
Multiple		85 (43.8)	8 (38.1)
Multiple with HR		79 (40.7)	8 (38.1)
HR Types	16	76 (39.2)	3 (14.3)
	18	14 (7.2)	1 (4.8)
	31	23 (11.9)	5 (23.8)
	33	6 (3.1)	0
	35	5 (2.6)	1 (4.8)
	39	10 (5.2)	3 (14.3)
	45	4 (2.1)	0
	51	10 (5.2)	1 (4.8)
	52	19 (9.8)	1 (4.8)
	53	9 (4.6)	2 (9.5)
	56	17 (8.8)	0
	58	10 (5.2)	1 (4.8)
	59	2 (1.0)	1 (4.8)
	68	3 (1.5)	0
	73	6 (3.1)	3 (14.3)
	82	2 (1.0)	0
Any HR		139 (71.6)	15 (71.4)
LR Types	6	8 (4.1)	0
	11	2 (1.0)	0
	40	3 (1.5)	0
	42	16 (8.2)	0
	54	11 (5.7)	1 (4.8)
	61	4 (2.1)	1 (4.8)
	70	7 (3.6)	0
	72	2 (1.0)	0
	81	5 (2.6)	0
CP6108		12 (6.2)	0
Any LR		22 (11.3)	2 (9.5)
UR Types	55	6 (3.1)	1 (4.8)
	62	9 (4.6)	1 (4.8)
	66	9 (4.6)	1 (4.8)
	67	6 (3.1)	1 (4.8)
	83	3 (1.5)	0
	84	9 (4.6)	2 (9.5)
Any UR		36 (18.6)	5 (23.8)

*corresponds to women at the initial study visit that do not have a history of colposcopic examination.

Figure 2.6 shows that there was a statistically significant increase in high-risk HPV positivity between cases of CIN 1 and CIN 2/3 ($p<0.05$), and also in the low-risk HPV positivity between cases with no CIN and CIN 1 ($p<0.05$). The proportion of multiple infections significantly increased in groups with histological abnormalities (no CIN to CIN 1) ($p<0.05$), but not as severity increased (CIN 1 to CIN 2/3) (Figure 2.6). The most prevalent high-risk HPV genotypes in this study show different patterns of HPV positivity among the different histological categories (Figure 2.7). Not surprisingly, HPV-16 positivity increased significantly from CIN 1 to CIN 2/3 ($p<0.05$), as was the case for genotypes 31 and 52, while the positivity of HPV-56 and HPV-39 decreased ($p<0.05$). No significant histological trends were identified for HPV-18 infections.

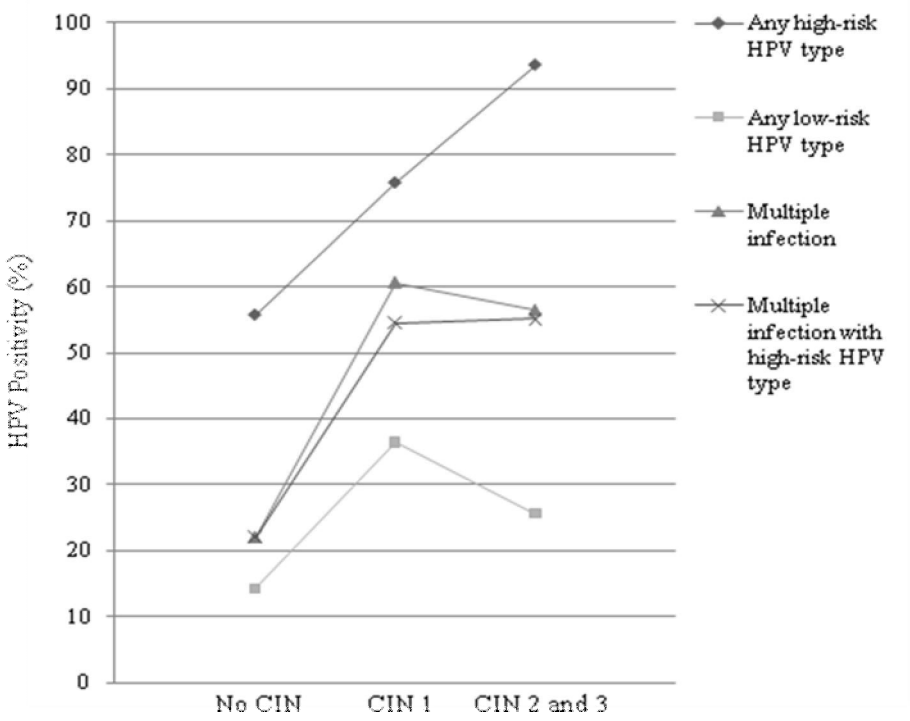

Figure 2.6: Prevalence of risk group specific HPV types, and multiple HPV infections by histological diagnosis. Percent HPV positivity indicates the proportion of samples that were positive for the given HPV category within the histological category.

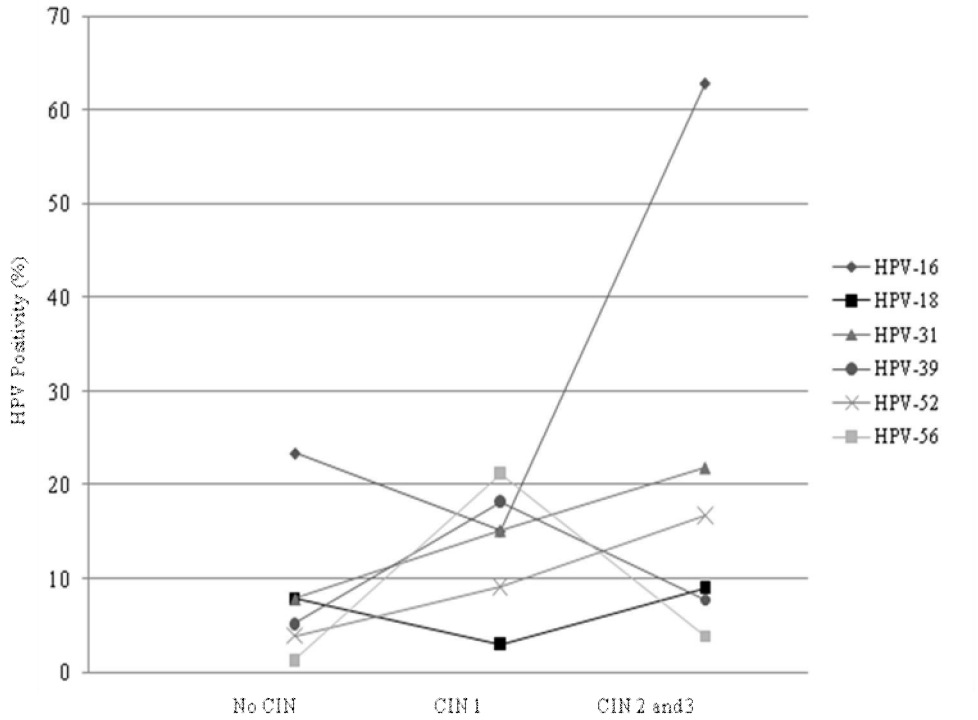

Figure 2.7: Prevalence of the most common HR-HPV types by histological diagnosis. The percent HPV positivity refers to the proportion that were positive for the given HPV category within the histological category.

The prevalence of multiple HPV infections changes with age and histological diagnosis (Table 2.3). Women 29 and under have a significantly higher proportion of multiple HPV infections than women over the age of 30, regardless of their histological diagnoses. In both age groups there is a significant increase in the proportion of multiple infections in patients with CIN ($p<0.05$, data not shown).

Table 2.3: Age-stratified comparison of multiple HPV infection prevalence in the presence or absence of CIN.

	Women 29 and under	Women 30 and over	p-value
	n=108	n=107	
No CIN			
Multiple	13	4	<0.05
Multiple with HR	12	4	
CIN 1/CIN 2/CIN 3			
Multiple	39	25	
Multiple with HR	39	23	<0.05
Total*			
Multiple	58	35	<0.05
Multiple with HR	56	32	<0.05

*Addition of No CIN and CIN1/2/3 cases do not equal the total because indeterminate specimens have been excluded from those categories but included in the total.

2.3.3 HPV Status of Women with ASCUS/LSIL Referral Pap Smears

Tables 2.4 describes the HPV status and histology results for women referred to the colposcopy clinic with abnormal cytology (ASCUS or LSIL); none of these women had a history of colposcopic examination. Histological analysis of these women revealed 51.7% (30/58) had no CIN, 29.3% had CIN 1 and 19.0% had CIN 3. Fourteen (46.7%) of the women with normal histology were HPV-negative, compared to 23.5% of the women with CIN 1 and 9.0% of the women with CIN 2/3; while, 90.9% (10/11) of the women with CIN 2/3 contained a high-risk HPV type, as compared to 64.7% of those with CIN1 and 43.3% of those with normal histology. Wait time was calculated as the number of months between the initial ASCUS or LSIL screening result and the colposcopy visit. This analysis revealed that those identified as CIN2/3 at the colposcopic visit waited a significantly lesser number of months than those with either no CIN or CIN 1 ($p<0.05$). Further, the wait time for those identified as no CIN or CIN 1 was significantly different.

Table 2.4: Analysis of HPV infections and the histological diagnosis in patients with clinical histories of persistent ASCUS/LSIL cytology results that were referred to the colposcopy clinic with ASCUS/LSIL cytology diagnosis.

Histology	n	% HPV+	% HR-HPV+	% LR-HPV+	Wait Time (months)[95% CI]
No CIN	30	53.3 (16/30)	43.4 (13/30)	20.0 (6/30)	17.7 [3.1-32.3]
CIN 1	17	76.5 (13/17)	64.7 (11/17)	23.5 (4/17)	19.6 [2.9-36.4]
CIN 2/3	11	90.9 (10/11)	90.9 (10/11)	27.3 (3/11)	13.6 [6.9-20.4]
Total	58	67.2 (39/58)	58.6 (34/58)	22.4 (13/58)	17.3[2.4-32.2]

2.3.4 HPV Status of Women Pre- and Post-Treatment for High-Grade Cervical Lesions

As stated previously, 31 of the 254 specimens submitted for the study were 6-month follow-up samples; this group of samples had study specimens collected at their initial visit and 6-month follow-up visit at the colposcopy clinic. Eight of the 31 6-month follow-up study samples (25.8%) submitted were treated for high-grade cervical lesions with loop electrosurgical excision procedure (LEEP) (Table 2.5). All of the samples were HPV positive at the initial visit and 87.5% (7/8) were infected with a high-risk HPV type. After treatment, all of the samples were negative for CIN (data not shown), and 6/8 (75.0%) of the samples were HPV negative. HPV positivity was retained in two of the samples. One of these had a new HPV infection and the other retained HPV positivity for two out of the three HPV types present prior to treatment.

Table 2.5: Analysis of infecting high-risk HPV types in specimens collected pre- and post- LEEP for high-grade cervical intraepithelial neoplasia (CIN).

Age	History of Colposcopy	Referral Pap Result	Pre-LEEP HPV	Post-LEEP HPV
41	no	HSIL	55, 67, 70	Negative
20	no data*	no info	16, 18, 56	73
39	yes	ASC-H	16, 35, 39	16, 35, 52
27	no	HSIL	39, 56, 66	Negative
16	no data*	LSIL	18, 67	Negative
35	no	HSIL	16	Negative
25	no	ASC-H	16, 31	Negative
29	no	ASC-H	16	Negative

*no data indicates that no clinical history was available

2.4 DISCUSSION

HPV genotyping and detection assays have been developed to supplement cervical cancer screening programs. These assays combined with the Pap smear have the ability to circumvent sensitivity and specificity issues associated with cytological analysis, while also providing knowledge of the regional HPV genotype prevalence and distribution within different populations of women. The sample population in this study is composed of women with a history of cytologic abnormalities, in comparison to a routine screening population, that are at a higher risk of developing cervical cancer. The main objective of this study was to characterize the HPV genotype prevalence and distribution within this population. The distribution of genotypes within this population could showcase the types of HPV that are most prevalent in the study region. Secondarily, the prevalence and distribution of HPV types in a subset of samples were analyzed in an attempt to characterize women with a history of persistent of ASCUS/LSIL cytology results that were referred for the same result. Also, HPV-infections and genotypes were identified in a small subset of the follow-up population (n=8) who were treated for the presence of a high-grade lesion using LEEP, with samples collected both pre- and post-treatment. Awareness of the most prevalent types of HPV in a high-risk population of women outlines the necessity for regional HPV genotyping studies in order to postulate the effectiveness of the vaccine.

Cervical cytology specimens were collected from 215 women, with a total of 254 specimens submitted. Initial specimens were used to determine the overall genotype prevalence and distribution of HPV in Edmonton and surrounding areas, within a population of women referred to colposcopy because of pre-existing abnormal cytology. Overall, 80.0% of the study population was HPV positive and 32 different HPV types were identified, similar to previous studies (11). In contrast, HPV positivity in routine screening populations may be 26% or less (12-14). The 6 most prevalent types of HPV found in the sample population were HPV-16, HPV-31, HPV-52, and HPV-39, HPV-42, and HPV-56. In agreement with other Canadian and internationally- based genotype prevalence studies, HPV-18 was not found to be among the most prevalent types (11, 15-20). Although, globally HPV-18 is responsible for a large proportion of

cervical cancer cases, the prevalence of HPV genotypes can vary considerably depending on the characteristics of the sample population and also the region or province an individual is located in (21). Within Canada alone, provincial variations in HPV genotype prevalence have been identified, and HPV-18 is not typically among the most prevalent high-risk HPV types (11, 16, 22-26); further, characterization of colposcopy-based patient populations may reveal different prevalence results.

As stated above, the sample population was divided into two groups: (1) those that had never had a colposcopic examination (n=194), and (2) those with a history of colposcopic examination for cervical lesions of varying degrees of severity (n=21). In colposcopy-based sample populations it is important to look at the clinical histories of the patients because women presenting at the clinic are heterogeneous and this ultimately leads to changes in HPV genotype prevalence. In stratifying the patient population based on colposcopic history, the most prevalent HPV types in women with no colposcopic history were identified as HPV-16, HPV-31, HPV-52, HPV-56, HPV-42 and HPV-18. In this analysis, HPV-18 was identified as one of the most prevalent types, and HPV-39 was no longer included. The group of women with a history of colposcopy had a much higher prevalence of HPV-39, and other high-risk HPV types not included in the group of women lacking colposcopic history. Overall, the 6 most prevalent types identified within the entire sample population are slightly different than those identified when the sample population is stratified according to colposcopic history and the presence of women with certain histories can skew the prevalence results to include or eliminate important HPV types.

HPV genotype prevalence and distribution varies not only geographically but also according to histological diagnosis. Women with CIN 2/3 had a significantly higher proportion of high-risk HPV infections, like HPV-16, -31, and -52, while women with CIN 1 had a significantly higher proportion of low-risk, HPV-56, and HPV-39 infections. A Quebec study recognized a similar trend; an increase in high-risk HPV type infections as histology becomes more severe (16). This decrease in low-risk HPV type infections is supported by the notion that they lack a role in the development of cervical cancer (16). The peak of HPV-56 prevalence in CIN 1 histology cases

is supported by a Saskatchewan study that showed similar variations in HPV-56 genotype prevalence among their histological categories (11). Similar to the decrease in low-risk HPV type prevalence, HPV-56 prevalence may decrease with increasing histology because of a lack of association with severe disease. Although the proportion of high-risk HPV types would be expected to increase as histology becomes more severe, it appears that not all high-risk HPV types are equally associated with severe disease.

The proportion of multiple HPV infections increased with the introduction of CIN and remained stable as histology became more severe. Research suggests that the proportion of multiple HPV infections is affected more by age than cytological diagnosis (10). However, studies are discrepant as to how the proportion of multiple infections changes with age (10, 20). Given that under the age of 30 most HPV infections, including those with multiple types, are transient and not clinically significant, one could expect to see a decrease in the proportion of multiple HPV infections over time. The aforementioned trend was identified in this study; women 29 and under had a significantly higher proportion of multiple infections then those women 30 and over. Therefore, it seems plausible to indicate, that the identification of multiple infections in women over the age of 30 may be more clinically significant than those identified in younger women.

Analysis of women with clinical histories of persistent ASCUS/LSIL results (n = 58) revealed a myriad of results. Histologically, more than half of them were normal (30/58), while another 19.0% (11/58) had high-grade cervical abnormalities requiring treatment. HPV testing further revealed that 67.2% of them were HPV-positive, with the majority of them infected with a high-risk HPV type (34/39), leaving just under one third of the specimens HPV-negative. Of the ASCUS/LSIL cases referred for colposcopy, a large number of women had negative histology and HPV results at the time of colposcopy and, with reflex HPV testing, could have continued with routine screening. The ACCSP guidelines, updated in 2009, indicate HPV testing on women over the age of 30 with ASCUS, and women over the age of 50 with LSIL (27). Patients proven to be HPV negative are returned to routine screening, while HPV positive patients would be referred for

colposcopy (27). In applying the above ACCSP guidelines to the above group of women and assuming their HPV status is consistent from the point of referral to the initial colposcopy visit where HPV genotyping was completed, 8 of 15 cases of ASCUS in women over 30, and no cases of LSIL in women over 50 would not have required colposcopy. In the absence of HPV genotyping assays, this reveals the potential for the over-screening of some women with ASCUS who may not have required it. Although this analysis appears to identify some cases of over-screening, these results need to be approached with caution as the HPV genotyping undertaken in this study was completed at a different point in the screening process than would occur according to the updated ACCSP guidelines. HPV genotyping in this study was completed at the initial colposcopy visit, where as the updated guidelines suggest genotyping should be completed at a screening visit prior to referral to the colposcopy clinic. It therefore may be problematic to assume that the HPV status of a patient would remain the same at both of these visits because exposure to new HPV types may occur.

Of the follow-up samples treated with LEEP, the HPV genotyping assay revealed one "treatment failure" that otherwise would have been undetected. In this case "treatment failure" is defined as the presence of the same HPV types both pre- and post-treatment, although in practice evidence of this alone would not indicate treatment failure, and lead to further treatment. The process of monitoring lesion re-development post-LEEP is confounded by the distortion of the transformation zone from the treatment procedure itself, which renders cytological analysis unreliable (28). HPV genotyping assays may be a good tool to supplement this surveillance as persistent HPV-type infections present both pre- and post-LEEP has been identified as a significant risk factor in the redevelopment of cervical dysplasia (28).

2.5 CONCLUSION

HPV genotyping assays have the ability to supplement many areas within a cervical cancer screening program. As demonstrated above post-LEEP testing can identify treatment failures; in routine screening it can discriminate abnormal cytology cases that need further

examination, and it can also provide information about the regional HPV genotype prevalence and distribution, which is important in monitoring vaccine effectiveness. In its current state, this study lacks generalizability because the majority of women enrolled in this study were below the age of 30, only few samples were collected, and they were representative of a high-risk population. Further research is still required to establish regional HPV genotype prevalence in the general population.

In June 2008, the Alberta government announced the implementation of a voluntary HPV vaccination program of females with the prophylactic vaccine, Gardasil® (Merck Frosst), which protects against HPV-16, HPV-18, HPV-6 and HPV-11 infections, in the absence of knowledge of regional HPV genotype coverage. As indicated in this study and many others completed throughout Canada, there are regional and provincial variations in HPV genotype prevalence (11, 16, 22-26). This study confirmed that HPV-16 is one of the most prevalent types in this region, followed by other high-risk HPV types that are not yet included in the vaccine. Although, the sample population is not representative of the general population, it gives the medical community a very good impression of the HPV types circulating in the community that are significant in the development of cervical disease, which should be targeted in the future.

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CHAPTER 3

The characterization of E6 and E7 viral mRNA expression as a biological marker of HPV infections

3.1 INTRODUCTION

HPV molecular diagnostic tests allow for the detection and genotyping of infecting HPV types within a cervical sample. These tests typically target HPV DNA to indicate infection, even though the presence of HPV DNA alone does not correlate well with the presence and progression of cervical disease. As stated previously, the majority of women will be infected with HPV while very few will develop cervical cancer (1). The detection of HPV RNA, specifically the transcripts of viral oncogenes E6 and E7, may be more predictive of viral oncogenesis and cancer progression (2). A direct link has been identified between the amount of E6 and E7 transcript and specific cytological results (3). Quantification of these transcripts is not only indicative of cytological diagnosis, but may also predict disease progression and regression of abnormal cases of undetermined significance.

The quantification of HPV-16 E6 and E7 transcripts in cervical infection is a more appropriate predictor of disease than current diagnostic markers**.** The main objective was to characterize a quantifiable association of HPV-16 E6 and E7 mRNA expression with the presence cervical cell dysplasia.

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines

Cell lines were obtained from the American Type Culture Collection (ATCC, Virginia, USA) to be used as positive and negative HPV-16 controls. CaSki (CRL-1550) cells were derived

from a metastatic deposit in the small intestine of an epidermoid cervical carcinoma and they contain integrated copies of HPV-16. C-33 A (HTB-31) cells were derived from a cervical carcinoma and do not contain any HPV DNA or RNA. CaSki cells were used as the positive HPV-16 control, and C33-A cells were used as the negative HPV-16 control. These controls were used for both the DNA and RNA experiments as studies show that CaSki cell cultures produce HPV-16 specific E6 and E7 mRNA, and C33-A does not (4, 5). The cell lines were cultured according to ATCC's specifications. Briefly, C33-A cells were grown in Eagle's Minimum Essential medium, and CaSki cells were grown in RPMI-1640 medium, both of which were supplemented with 10% fetal bovine serum and 0.4% gentamicin. The cells were maintained in an environment of 5% CO_2 at 37 °C until passaging. The cells were harvested when confluent using 0.25% trypsin-0.03% EDTA. Following incubation at room temperature until the cells were detached, they were re-suspended in their corresponding media and sub-cultivated at a ratio of 1 to 8. The remaining cells were counted using a hemocytometer and preserved in PreservCyt® at a concentration of 10^6 cells/mL. The preserved cells were maintained at room temperature, as per the manufacturers' specifications for up to 6 weeks, to mimic the condition of the patient samples in preservative.

3.2.2 Primer Sequences

The desired target sequence of the HPV genome was chosen because it spanned a region that was conserved in both the HPV-16 E6 and E7 transcripts (Figure 3.1). The primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify a 123 base pair fragment between nucleotides 104 and 226. E6 and E7 proteins are translated from bicistronic pre-mRNA which encodes three exons and two introns (6, 7). The E6 protein is translated from the full length, unspliced E6/E7 pre-mRNA, and the E7 protein is translated from the splice product E6*I, which lack nucleotides 226 through 409 (6). An E6/E7 oligonucleotide primer pair was selected against the region spanning nucleotides 104 through 226 to allow quantification of both gene products inclusively. Primers targeting S9 rRNA as the

endogenous control of RNA quantification had been previously published (3) (Table 3.1).

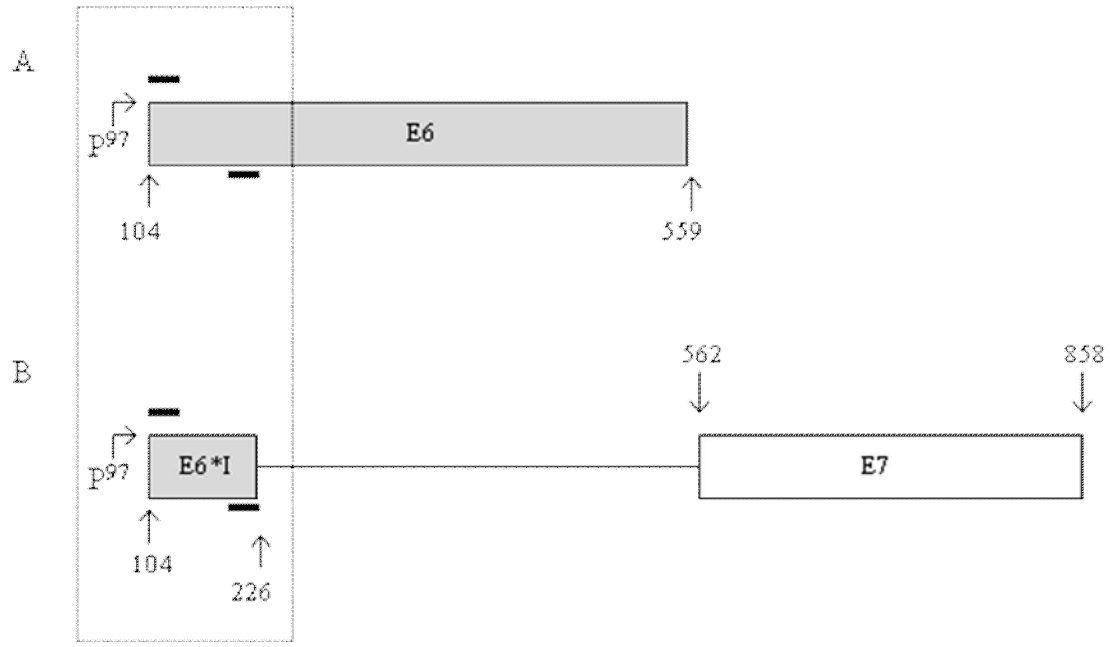

Figure 3.1: The conserved E6/E7 primer sequence in HPV-16 E6 and E7 transcripts. (A) A representation of the mRNA, which leads to the translation of the E6 protein. The box represents the full length E6 mRNA which is required in the translation; no alternative splicing occurs. P^{97} identifies the promoter that is used for transcription initiation. (B) A representation of the mRNA used in the translation of the E7 protein. In this diagram the boxes represent exons and the line represents an intron. Alternative splicing of the mRNA leads to the production of E6*I, which is a truncated section of the E6 gene containing nucleotides 104 through 226. This splicing leads to the effective translation of the E7 protein. The dashed box around (A) and (B) indicates the conservation of the mRNA sequences in both mRNAs, stretching from nucleotide 104 through 226. Forward and reverse primers (bolded, black lines) directed against the conserved E6/E7 sequence are indicated at their approximate nucleotide positions in both (A) and (B). Adapted from Tang, *et al.* (6).

Table 3.1: Primer pair sequences used for PCR and real-time PCR.

Primers	Sequence
E6/E7 Forward	5'-AAT GTT TCA GGA CCC ACA GG-3' (nt 103-122)
E6/E7 Reverse	5'-CAA CAG TTA CTG CGA CGT GA-3' (nt 206-225)
S9 Forward *	5'-ATG CGC CAG CGC CAT A-3' (nt 419-434)
S9 Reverse*	5'-TCA ATG TGC TTC TGG GAA TCC-3' (nt 484-504)

* S9 primer pair has been previously published by Wang-Johanning, *et al.* (2002) (3).

3.2.3 E6/E7 Plasmid Construction

In order to build a plasmid construct containing the desired E6/E7 sequence, HPV-16 DNA must be extracted, amplified via PCR, purified and then transformed into chemically competent cells, at which once a positive clone is verified will be used to make plasmid and glycerol stocks for subsequent use and experimental procedures. Total DNA was extracted from CaSki and C33-A cells using a DNEasy Blood and Tissue Kit (Qiagen) following the spin column protocol outlined as "Total DNA from Animal or Blood Cells" in the product insert. The quantity and quality of DNA was determined spectrophotometrically. The total DNA was amplified via PCR using the primer pair directed against E6/E7 mRNA (Table 3.1). The primer pair can be used in the amplification of both E6/E7 mRNA and DNA because it targets a sequence that is the same in both nucleic acid products. The amplification reaction was performed in a 50 µL volume containing 1X High Fidelity PCR Buffer (Invitrogen), 0.2 mM dNTP mixture, 2 mM $MgSO_4$ (Invitrogen), 1.0 µM forward primer, 1.0 µM reverse primer, Platinum® *Taq* High Fidelity (Invitrogen), nuclease-free water, and 1 µL template. Amplification was completed using a 96-well GeneAmp® PCR System 9700 (Applied Biosystems) with a gold-plated silver sample block using the following cycle parameters: 94 °C for 30 seconds, 25 cycles of 94 °C for 15 seconds, 52°C for 15 seconds, and 68 °C for 1 minute, followed by 72 °C for 7 minutes, and a hold at 4 °C. A 2.0% agarose gel, run at 120 V for 45 minutes was used to confirm amplification of the desired product (Figure 3.2). One band approximately 125 base pairs (bps) in length were produced from

this reaction mix; the expected product size from this primer pair is 123 bps.

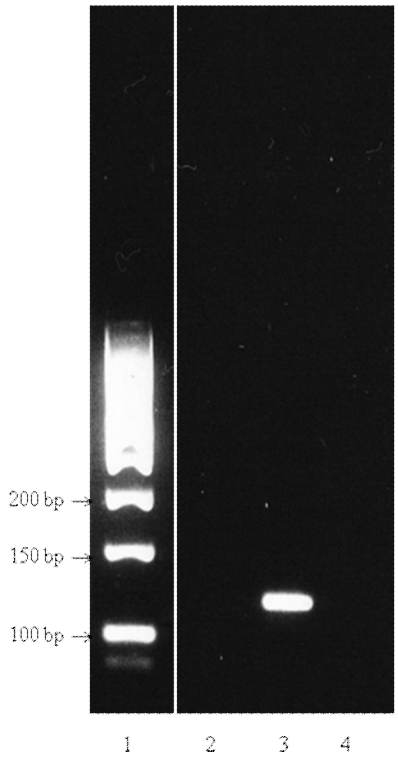

Figure 3.2: Amplification products from the E6/E7 primer pair. Lane 1 contains a 100 bp DNA ladder. Lane 2 corresponds to a no template control. Lane 4 corresponds to the negative control reaction containing DNA from C33-A cells. Lane 3 corresponds to the positive control reaction containing DNA from CaSki cells.

The desired fragment was purified using a QiaQuick Gel Extraction Kit (Qiagen), and then cloned into a pCR® II-TOPO® plasmid with dual promoter capabilities via a TOPO TA Cloning Reaction (Invitrogen), following the manufacturer's protocol. In brief, the PCR product was mixed with a salt solution, water and the vector at room temperature and left to incubate for 5 minutes. The reaction mix was then combined with One Shot® TOP 10 Chemically Competent Cells and put on ice for 30 minutes. The cells were heat shocked at 42 °C, returned to ice and then incubated at 37 °C for 1 hour with horizontal shaking (200 rpm). The transformation reaction was spread on a selective Luria-broth plate, containing X-gal, and incubated at 37 °C overnight. Transformed (white) colonies were subcultured in LB medium overnight and used as template for colony PCR. Colony PCR was completed using the same PCR parameters and reaction mix components as described above. The PCR products were resolved on a 2.0% agarose gel, run at 120 V for 45 minutes, to confirm the presence of the desired fragment of approximately 120 base pairs. The plasmid DNA containing the target sequence was purified using the Qiaprep Spin Miniprep Kit (Qiagen) and stored at -20 °C. The quantity of plasmid DNA recovered was established using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific). A glycerol stock of the positive clones was also prepared and frozen at -80 °C. To confirm the presence of the sequence and also its orientation in the construct, plasmid DNA was sequenced in both directions using M13 forward and reverse primers on the Beckman Coulter CEQ2000XL DNA Sequentator (Figure 3.3). Sequence results revealed the orientation of the target sequence within the plasmid and based on this *in vitro* transcription was primed using the T7 promoter.

Figure 3.3: A representation of the 123 base pair HPV-16 E6/E7 target sequence within the pCR®II-TOPO® plasmid. The highlighted grey nucleotides indicate the sequences required at the 3' ends for TA cloning. The nucleotides within the dashed boxes are the E6/E7 forward and reverse primer sequences and the nucleotides between these dashed boxes are the target sequence. Arrows on the plasmid vector indicate *FspI* cleavage sites at nucleotides 569, 1592, and 2738.

3.2.4 *In Vitro* Transcription

The *in vitro* transcription reaction allows for the production of target RNA from the plasmid that can then be used for RNA standards in the production of the standard curve for absolute quantification. The E6/E7 plasmid was digested to further confirm the presence of the target sequence based on expected band sizes, and also to linearize the plasmid for *in vitro* transcription. The plasmid was linearized using restriction enzyme (RE), *FspI* (New England BioLabs® Inc.). This enzyme was chosen because it produced a distinct number of fragments with blunt ends and it also did not cleave within the target sequence. The RE digestion reaction mix was setup according to the product specifications and incubated at 37 °C for one hour. RE digestion was terminated through the addition of ethanol and incubation at -20 °C for 15 minutes. The linearized DNA was pelleted by centrifugation (15000 rpm, 15 minutes), re-suspended in TE buffer at a concentration of 0.5-1 µg/µL, and stored at -20 °C. Complete linearization of the plasmid with *FspI* was analyzed using a gel electrophoresis (1% agarose gel, 100V for 60 minutes) (Figure 3.4). The bands correspond to DNA fragments that were approximately 1000 bps, 1200 bps, and 2000 bps in length. According to the restriction map three bands were expected to be produced, which measure 1023 bps, 1146 bps, and 1804 bps each; the largest fragment would contain the target sequence. This gel confirms the presence of the desired target fragments and also the complete digestion of the plasmid with *FspI*.

In vitro transcription was completed using the MEGAscript® Kit (Ambion), according to the manufacturer's specifications. A reaction mix containing ribonucleotide solutions (ATP, GTP, CTP, and UTP), 1X reaction buffer, an enzyme mix (RNA polymerase), the linearized template DNA and water was assembled at room temperature, mixed, and incubated at 37 °C for 6 hours. The resulting RNA was purified using the MEGAclear™ Kit (Ambion) according to the manufacturer's specifications. The presence of the desired RNA fragment (~2000 base pairs) was confirmed using denaturing agarose gel electrophoresis (Figure 3.5).

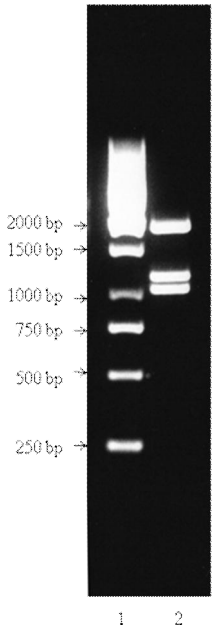

Figure 3.4: Gel containing products of FspI digested plasmid in preparation for *in vitro* transcription. Lane 1 showcases the 1 kb DNA ladder. Lane 2 showcases the 1023, 1146, and 1804 bp bands produced through FspI digestion.

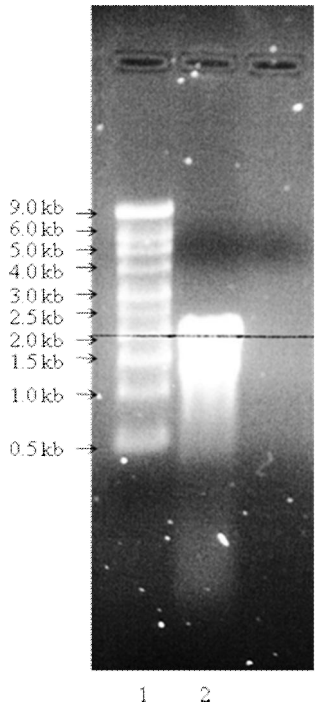

Figure 3.5: A denaturing agarose gel confirmed the presence of RNA post-*in vitro* transcription. Lane 1 contains a 1 kb RNA ladder. Lane 2 showcases the RNA fragment, approximately 2000 base pairs in length, produced from the *in vitro* transcription reaction.

The quantity of complementary RNA (cRNA) produced by the transcription reaction was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. The copy number of the E6/E7 cRNA was calculated using the following formula, which has been previously published (8):

$$\text{Copy Number (copies/µL)} = \left(\frac{\text{g/µL cRNA}}{\text{Transcript length in nucleotides x 340}} \right) \text{x } 6.022 \text{ x } 10^{23}$$

Whereby, 340 = molecular weight for RNA, transcript length in nucleotides = 1766 bp, and 6.022×10^{23} = Avogadro's Constant.

The cRNA was diluted in nuclease-free water into aliquots containing 10^9 copies/µL and stored at -80 °C for subsequent use.

3.2.5 Probe Sequences

A TaqMan® TAMRA™ (carboxytetramethylrhodamine) probe (Applied Biosystems) labelled with 6-carboxyfluorescein (FAM™) was designed to anneal within the target, from nucleotide 141 to 164. OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to verify the absence of self-complementarity (i.e. hairpin formations) within the probe sequence and a NCBI BLAST search confirmed sequence uniqueness with HPV-16 E6/E7. A TaqMan® TAMRA™ probe (Applied Biosystems) labelled with VIC® was designed previously to anneal to the endogenous control S9, from nucleotides 419 to 504 (3) (Table 3.2).

Table 3.2: TaqMan® sequences used for real-time PCR.

Probe	Sequence (5'-3')
E6/E7	6FAM™-CAC AGT TAT GCA CAG AGC TGC AAA-TAMRA™
S9*	VIC®-AGC AGG TGG TGA ACA TCC CGT CCT T-TAMRA™

* S9 probe sequence was previously published by Wang-Johanning, *et al.* (2002) (3)

3.2.6 RNA Extraction and DNase treatments

RNA was extracted and reverse transcribed from clinical specimens, RNA standards, and HPV controls simultaneously and in duplicate for each PCR experiment. Total RNA extraction was completed using patient samples and controls in preparation for the reverse transcription reaction. RNA work was completed in a designated room maintained RNase-free. Surfaces and equipment were consistently treated with RNaseZap® (Ambion) prior to use and consumables were handled with RNase-free technique. Total RNA extraction of HPV-16 positive (confirmed by genotyping in Chapter 2) patient specimens and controls were completed using the RNAqueous® Kit (Ambion). One mL of each specimen, containing an unknown number of exfoliated cells, was aliquoted and spun at 13000xg for 20 minutes. One mL of each control, at a concentration of 10^6 cells/mL was also aliquoted and spun as indicated previously. The resulting pellets were lysed, washed and eluted. TURBO™ DNase treatment was completed twice according to the rigorous DNase treatment instructions and also the TURBO DNA-free™ Second Digest Protocol (http://www.ambion.com/techlib/append/supp/digest.html). The resulting total RNA was quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. Any remaining total RNA not used for reverse transcription was stored at -80 °C.

3.2.7 Reverse Transcription

Reverse transcription was completed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction mix contained 1X RT buffer, 5X dNTP Mix (100 mM), 1X RT random primers, MultiScribe™ reverse transcriptase, RNase inhibitor,

nuclease-free water and 10 µL of total RNA. The reaction was completed using a 96-well GeneAmp® PCR System 9700 with a gold-plated silver sample block using the following cycle parameters: 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes, and 4 °C infinitely. The resulting cDNA was purified to remove unincorporated nucleotides using NucAway™ Spin Columns (Ambion) and quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer. The cDNA was then diluted to a concentration of 8.0 ng/µL according to the manufacturer's specifications for use in real-time PCR. Remaining cDNA not used for real-time PCR was stored at -20 °C.

3.2.8 Real-Time PCR

Following reverse transcription, samples and controls were diluted and the standard dilution series was setup to contain dilutions of 10^8 copies/µL and 10 fold dilutions ranging from 10^5 to 10^0 copies/ µL. The use of this dilution series in HPV RNA quantification experiments had been previously published (9). The reaction mix contained 1X TaqMan Gene Expression Master Mix (Applied Biosystems), 1X E6/E7 assay mix (18µM forward and reverse primers, and 5µM E6/E7 6-FAM™ labelled TaqMan® probe), 1X S9 assay mix (18 µM S9 forward and reverse primers, and 5 µM S9 VIC®-labelled TaqMan® probe), 20 ng of sample or control, and water, combined in a final reaction volume of 25 µL. The reaction was setup using a MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems) and was run on a StepOnePlus™ Real-Time PCR System using the following cycle parameters: 60 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A standard curve was constructed using the points from the standard dilution series, and the copy number (copies/ µL) of E6/E7 mRNA within the samples and controls were extrapolated from this.

3.2.9 Normalization of E6/E7 mRNA

Absolute quantification is accomplished by using a standard curve to determine the copy number of the gene of interest, while relative quantification is accomplished by relating the

expression of the gene of interest to the expression of another gene (10). Absolute quantification was completed in this study because the desired outcome was a copy number for the E6/E7 mRNAs as opposed to an amount relative to the expression of another gene. Further, normalization was completed to ensure the amount of E6/E7 mRNA quantified was restricted to only those cells expressing it. Normalization was accomplished using a previously described method that utilizes flow cytometry to detect HPV-infected cervical epithelial cells in the total cell population (11). The HPV OncoTect Test Kit (Invirion Diagnostics) was used for this purpose. CaSki and C33-A cell lines were used as the positive and negative HPV-16 controls. The samples and controls were adjusted to a concentration of 10^6 cells/mL, spun at 1000 x g for 5 minutes, washed several times with PBS, and permeabilized at room temperature for 1 hour. Next, the cells were hybridized with a HPV hybridization cocktail composed of 5'-, 3'- fluorescein isothiocyanate (FITC) labelled oligonucleotide probes in a 43±1 °C water bath. Lastly, the cells were washed to remove residual unbound probe. The suspension of cells was analyzed using the Becton Dickinson Benchtop FACSort (BD Biosciences) and a minimum of 5000 events were collected. The different populations of cells were gated, or separated, based on forward and side scatter properties, and the median fluorescent intensities (MFI) were obtained for each individually gated population.

3.2.10 Absolute Quantification of HPV-16 E6/E7 mRNA

The absolute quantity of E6/E7 mRNA was obtained by adjusting for the proportion of cells expressing E6/E7 mRNA within a given sample. The absolute quantity of E6/E7 mRNA in a given sample corrected for the proportion of cells expressing E6/E7 mRNA can be determined by multiplying the amount (copies/µL) of E6/E7 mRNA obtained through real-time PCR with the proportion of cells expressing E6/E7 mRNA determined through flow cytometry. Correlations in the amount of E6/E7 mRNA present between different grades of cytology in HPV-16 positive specimens were made.

3.3 RESULTS

3.3.1 Optimization Experiments

Initial experiments were completed to optimize the experimental conditions when working in multiplex (both 6-FAM™ and VIC® probes and primer pairs are present) to determine the effects of working in multiplex on the threshold cycle, or $C_{t,}$ values, as compared to working in singleplex (either 6-FAM™ or VIC® probe and their analogous primer pair is present). The threshold cycle is the cycle number during the PCR reaction when the sample fluorescence exceeds the background fluorescence indicating true amplification within the sample (12). In the standard dilution series (10^7 to 10^{12} copies/µL) working in multiplex produced a large shift in the C_t values for the two highest dilution points (10^{11} and 10^{12} copies/µL) and only small variations in the C_t values for the lowest dilution points (10^7 to 10^{10} copies/µL) (data not shown).

Verification was completed to determine the amount of E6/E7 mRNA in the CaSki and C33-A cells to clarify whether or not the highest dilution points would need to be included in the dilution series (Figure 3.6). Standard dilution series were made to include dilutions ranging from 10^7 through 10^{12} copies/µL. The HPV-16 positive controls fell within this range, producing an average of 7.52×10^7 copies/µL. This confirmed previous reports indicating that the standard curve should contain dilution points ranging from 10^8 to 10^5 through 10^0 copies/µL (9). Further optimization for the highest dilution points (10^{11} and 10^{12} copies/µL) of the standard dilution series were not completed because they were not included in the final dilution series.

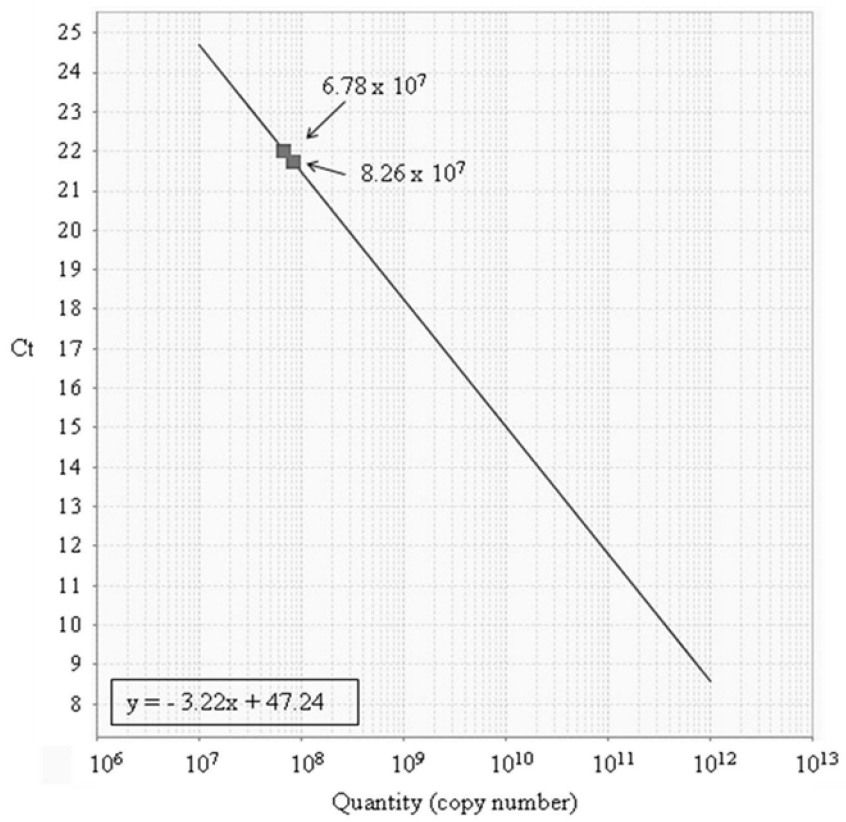

Figure 3.6: A standard curve produced by a standard dilution series ranging from 10^7 to 10^{12} copies of E6/E7 mRNA/µL. The points represent where the CaSki replicates fell within the dilution series.

Experiments were completed to determine the effects of dilution on the RNA standards. It was initially suggested that the RNA standards should be diluted prior to reverse transcription, however working with seven dilution points in duplicate was incredibly time consuming and wasteful. A comparison of diluting before reverse transcription and after reverse transcription was completed to determine its effects on the standard curve (Figure 3.7). An R-squared (R^2) value of 0.99 was obtained as an average for both dilution replicates. The R^2 value is a measure of the closeness of each data point to the standard curve, and a value close to 1 indicates there is little variation of the points from the curve. In real-time PCR, the desire is to have R^2 values > 0.99 because this will give the most reliable estimates of sample quantities extrapolated from the curve. Visual inspection of the standard curves reveals minor, negligible variations among the points produced in each standard dilution series. This indicates that the point at which dilution occurs does not affect the outcome of the standard curve. From this point on, the RNA standards were diluted post-reverse transcription because of ease of procedure and conservation of materials.

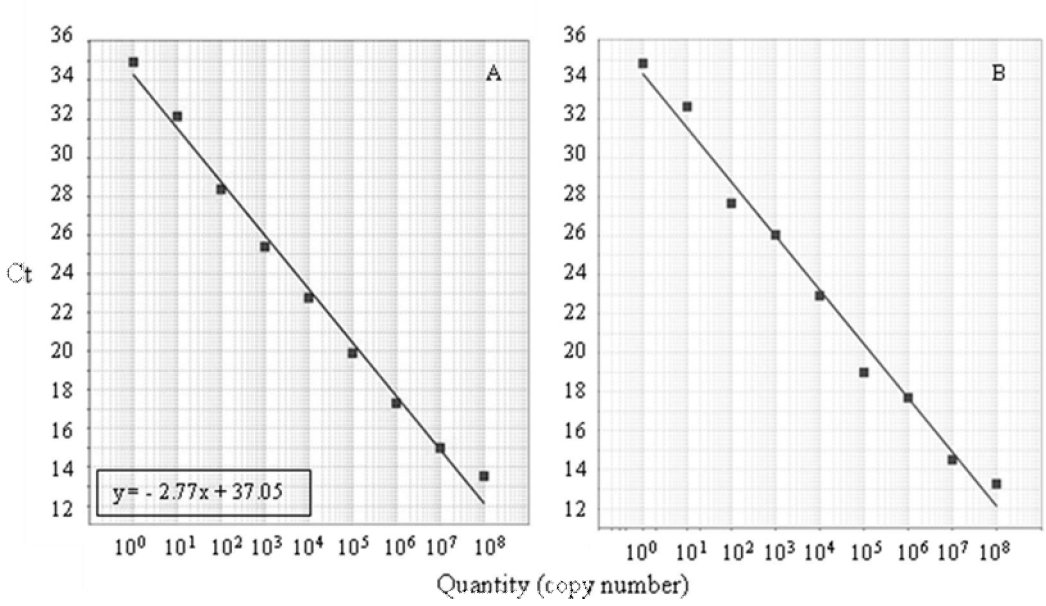

Figure 3.7: A comparison of the standard curves produced by standard dilution series (10^0 to 10^8 copies/µL) diluted prior to (A) or after reverse transcription (B). The points indicate the dilution points within the standard dilution series.

3.3.2 DNase Treatment Optimization

During the initial runs of patient samples, an amplification curve was seen in the no reverse transcriptase (RT) controls. A no RT control was assembled to identify contaminating DNA within the total RNA extracts. The reaction mix contains all of the elements required for reverse transcription minus the reverse transcriptase or the RNA extract is added directly to the PCR master mix; in either case there should be no DNA template in the reaction. The no RT controls in this study, showed amplification which corresponded to the presence of HPV E6/E7, while no S9 amplification was seen (Figure 3.8). Further, this amplification was only seen in HPV-16 positive specimens and controls (Figure 3.8). A number of different DNase treatment scenarios were completed to try to eliminate this spurious amplification. DNase concentrations were increased, and with each consecutive increase, amplification was seen at a later C_t value (Figure 3.9A). Then DNase incubation times and concentration were increased simultaneously, and again amplification was seen at a later C_t (Figure 3.9B). This DNase treatment would mimic treatment that would need to be completed on the samples, controls, and standards that had been previously treated with DNase. For both of these scenarios amplification shifted to a later C_t (>35) but was not completely eliminated (Figure 3.9A and 3.9B). Updated protocols included with the TURBO DNA-free™ kit outlined a new rigorous DNase treatment and also a procedure and buffer recipe for a repeat DNase digest (http://www.ambion.com/techlib/append/supp/digest.html). The updated methodology was followed, and as seen with the aforementioned scenarios, amplification corresponding to the presence E6/E7 DNA in the no RT controls was still present (C_t>35) (Figure 3.10).

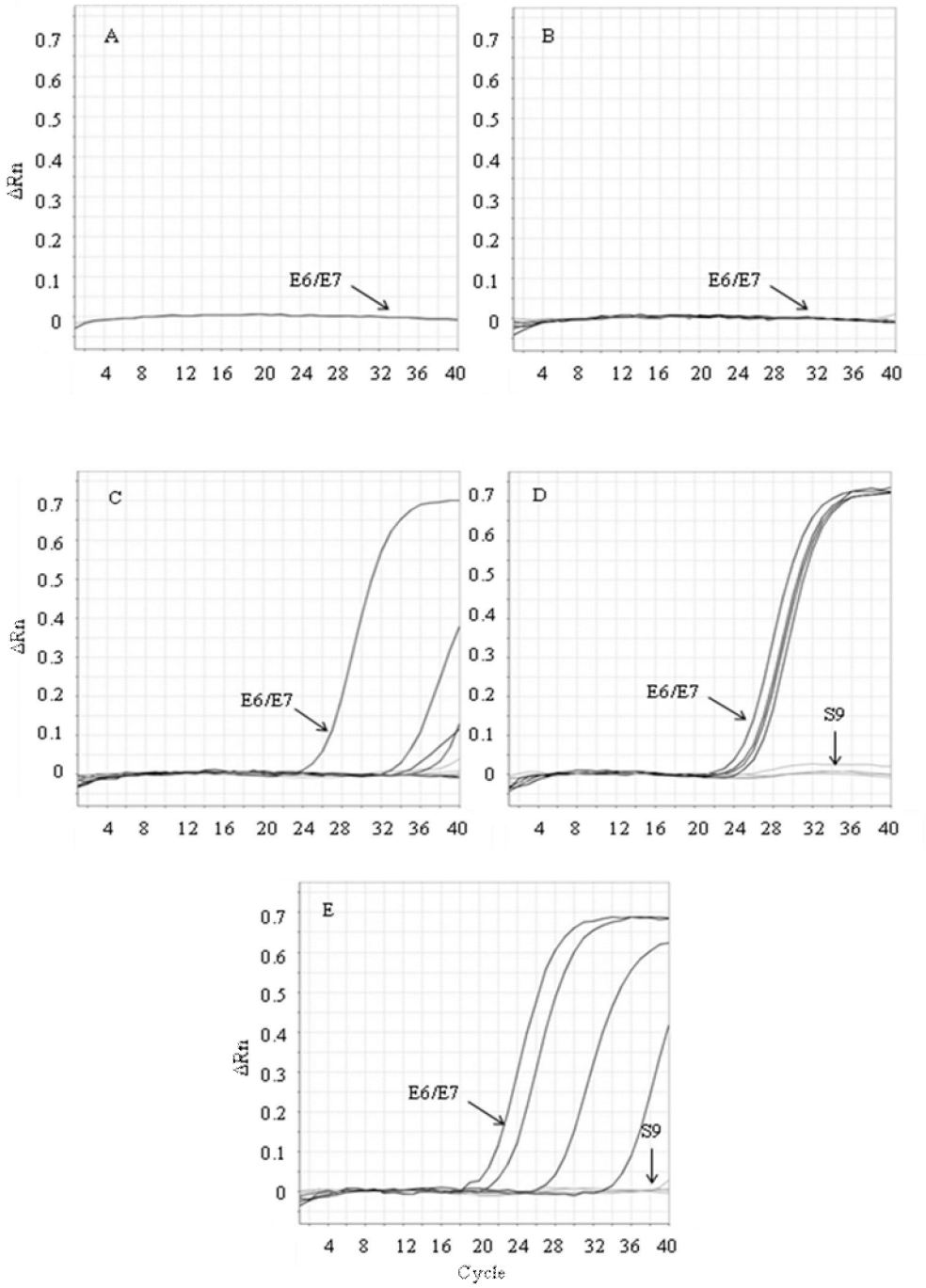

Figure 3.8: A comparison of amplification plots produced by no RT controls corresponding to the no template control (A), C33-A cells (B), HPV positive patient samples (C), 10^9copies/µL standard RNA dilution point (D), and CaSki cells (E). The grey curves (D and E) were produced by S9 amplification; the black curves (on all plots) were produced by E6/E7 amplification.

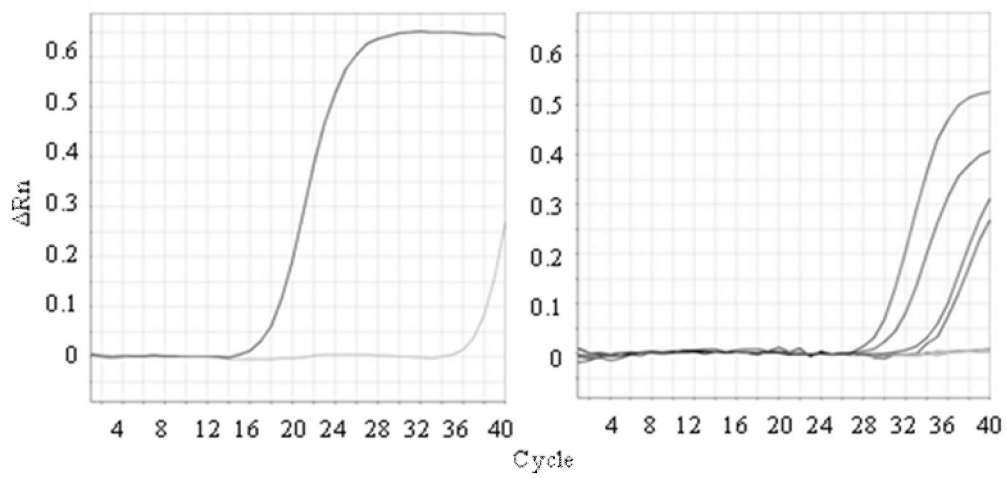

Figure 3.9A: A comparison of amplification plots produced by CaSki cells that have not been exposed to DNase (left) and those that have been exposed to increasing concentrations of DNase (right). The grey curves were produced by S9 amplification. The black curves were produced by E6/E7 amplification.

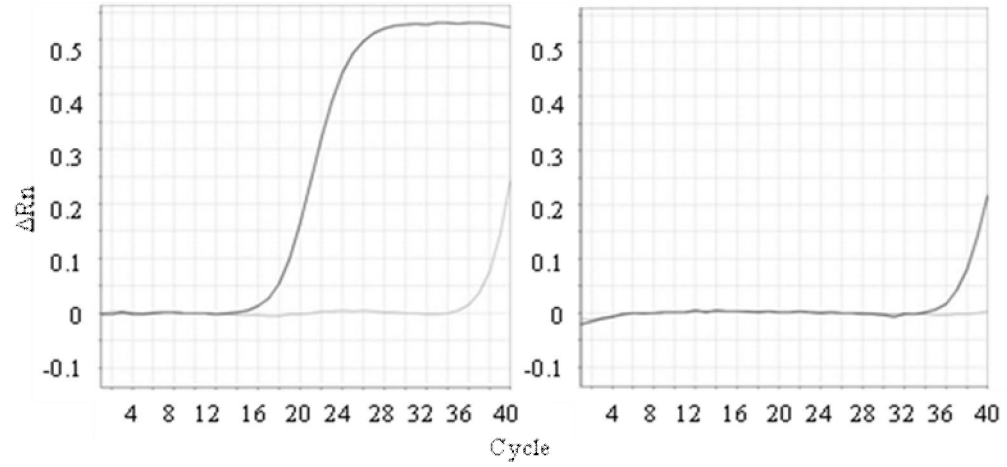

Figure 3.9B: A comparison of amplification plots produced by CaSki cells that have not been exposed to DNase (left) and those that have been exposed to an increased concentration of DNase for an increased incubation time (right). The grey curves were produced by S9 amplification. The black curves were produced by E6/E7 amplification.

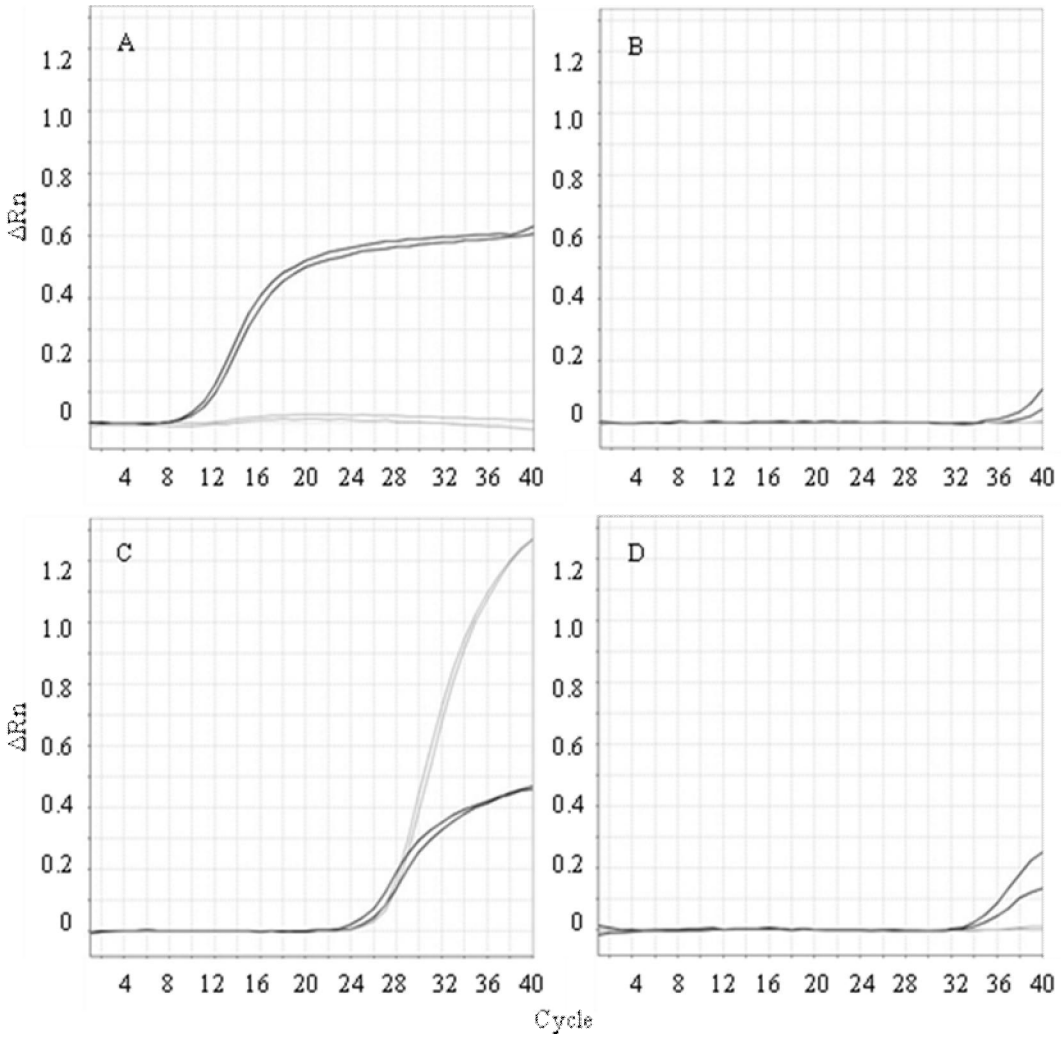

Figure 3.10: A comparison of amplification plots produced by the 10^9copies/µL standard RNA dilution point (A and B) and CaSki cells (C and D) that have not been treated with DNase (A and C) and those that have been treated with DNase according to the updated protocol (B and D). The grey curves were produced by S9 amplification. The black curves were produced by E6/E7 amplification.

The no RT products that were consistently seen in the HPV positive samples and CaSki cells were resolved using gel electrophoresis to confirm the product identity (Figure 3.11). The C33-A cells cDNA showed the presence of one band, around 85 bps, which corresponds to S9 (see lane 6A on Figure 3.11). Its analogous no RT control, did not have any bands. All of the patient cDNA samples have products that corresponded to S9, but not E6/E7 (see lane 2A, 3A, 4A, and 5A on Figure 3.11). The CaSki cells cDNA had two bands, one corresponding to S9 (~85 bps), and another corresponding to E6/E7 (~120 bps) (see lane 7A on Figure 3.11). Its analogous no RT control showed the presence of one band at approximately 120 bps, but the size of this band was not equivalent to the band corresponding to E6/E7 in the cDNA (see lane 7B on Figure 3.11). Patient samples that were HPV-16 positive show a similar band corresponding to this "E6/E7" product in their no RT controls (see lane 2B, 4B and 5B on Figure 3.11). All of these bands were slightly larger than 120 bps, and the difference in size compared to the E6/E7 band in the CaSki cell cDNA was visually distinguishable. However the confirmation of this amplicon as the target sequence in the no RT control was supported by the need for both E6/E7 primer pair and probe hybridization.

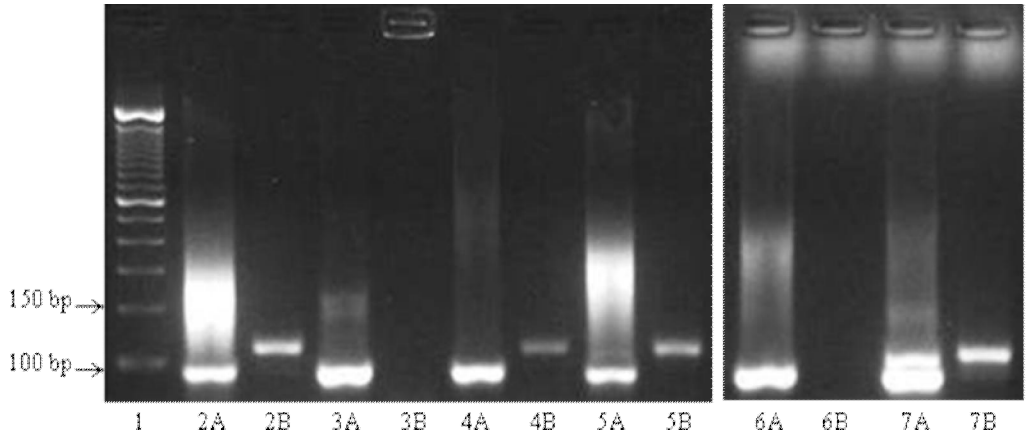

Figure 3.11: A gel resolving the products produced by real-time PCR in the samples (A) and analogous no RT controls (B). Lane 1 contains a 100 bp DNA ladder. Lanes 2 through 5 contain the products produced by four different patients. Lanes 6 and 7 contain the products from C33-A and CaSki cells, respectively.

3.3.3 Optimization of Real-Time PCR

Experimental conditions, including the annealing temperature and input template amount, were altered to increase the ΔC_t between the specimens and the no RT controls. Bustin (2004) suggests that when the C_t for a sample differs by more than 5 cycles relative to its no RT control the reaction is not considered a false positive (12). Thus, the E6/E7 curves in the HPV positive samples and controls needed to amplify at least 5 cycles earlier than their corresponding no RT controls, to produce a ΔC_t of no less than 5. The ΔC_t was calculated as the difference between the C_t values produced by E6/E7 amplification in the sample and it's no RT control. A decrease in the annealing temperature decreased the ΔC_t (Figure 3.12). Further, an increase in the amount of input template shifted the amplification curve corresponding to E6/E7 cDNA to an earlier cycle, increasing the ΔC_t (Figure 3.13).

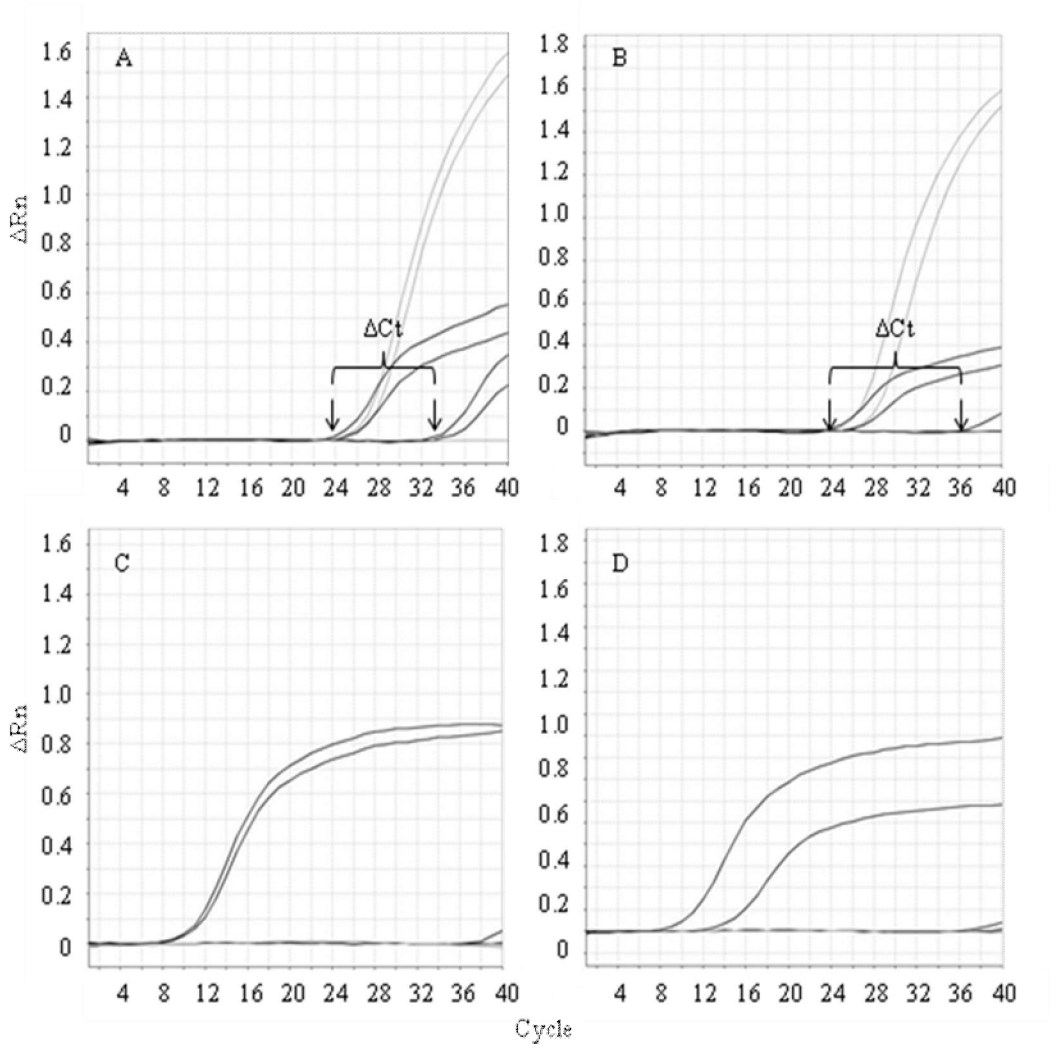

Figure 3.12: A comparison of amplification plots produced when the annealing temperature was changed from 60 °C (B and D) to 50 °C (A and C). Plots A and B were produced by CaSki cells, and C and D were produced by the 10^9 copies/µL standard RNA dilution point. Each plot includes amplification from samples and their analogous no RT controls. The arrows (in A and B) at the earlier C_t's correspond to amplification of sample cDNA, while the arrows at the later C_t's correspond to amplification in the no RT controls. The ΔC_t for E6/E7 amplification was calculated using the aforementioned C_t values and is labelled on each of the CaSki cell plots (A and B). The grey curves (seen on plots A and B) were produced by S9 amplification. The black curves (seen on all plots) were produced by E6/E7 amplification.

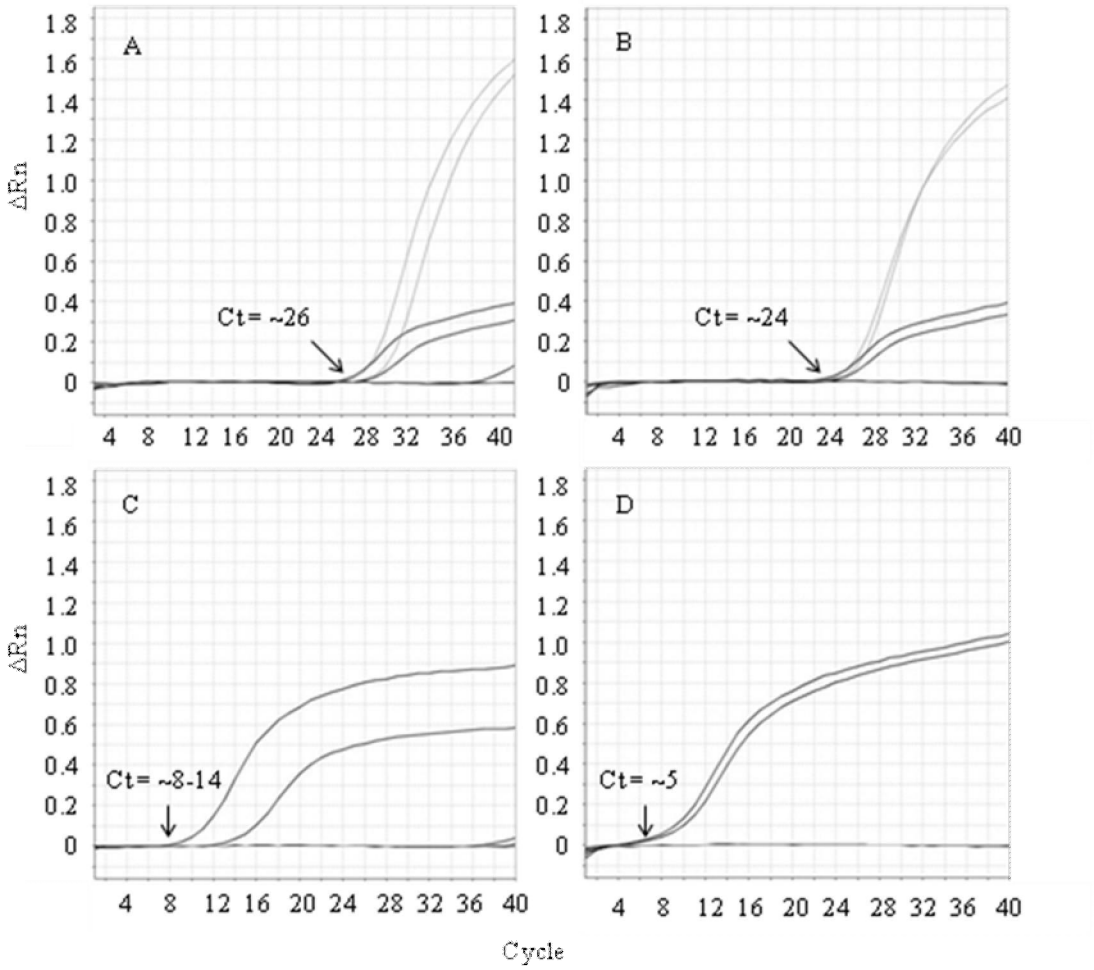

Figure 3.13: A comparison of the amplification plots produced when the input template was increased from 2.5 (A and C) to 7.5 µL (B and D). Plot A and B were produced by CaSki cells, and C and D were produced by the 10^9 copies/µL standard RNA dilution point. Each plot includes amplification from samples and their analogous no RT controls. C_t values which correspond to E6/E7 amplification of the sample cDNA are indicated on the plots. The grey curves (seen on plots A and B) were produced by S9 amplification. The black curves (seen on all plots) were produced by E6/E7 amplification.

3.3.4 Comparisons of Cytology and E6/E7 Expression

Experimentation revealed HPV controls and samples consistently show amplification corresponding to the presence of S9 however amplification in the patient samples typically occurs at a later C_t. In order to assume that the samples are adequate for analysis, and comparable to the controls, the amplification of S9 in the samples should be occurring at similar C_t's. In preparation of the HPV controls for RNA extraction, it was always ensured that 10^6 cells/mL were added into each reaction. This same measure was not taken for patient samples because there has been no adequate, bench-top technique identified to count the number of exfoliated cells within them. Due to the large amount of variability with regard to cell count between patient samples it does not seem feasible to assume that adding the same volume of patient sample to each reaction will result in sample adequacy. In an attempt to control for the disparity in the number of cells between the cervical specimens, patient samples were assumed to be adequate for analysis when S9 amplification was occurring at the same or earlier C_t than the controls in the same reaction; inadequate samples, with S9 amplification at later C_t than the controls were excluded from further analysis. Based on the aforementioned protocol, with regards to input template amount, for shifting the E6/E7 amplification curve to an earlier C_t, a larger volume of sample was added into the total RNA extraction reaction (4 mL vs. 1 mL) (12) (Figure 3.14). All patient samples in this analysis were HPV-16 positive, with high-grade cytology (ASC-H, or HSIL). Amplification plots indicated that this increase produced S9 C_t values in many of the samples that were less than or equal to that of the HPV controls (Figure 3.14). No E6/E7 amplification was seen in any of the patient samples or CaSki cells. Due to the age of these samples this experiment was repeated with samples that had been in the preservative for a shorter duration of time (data not shown), and the results were similar.

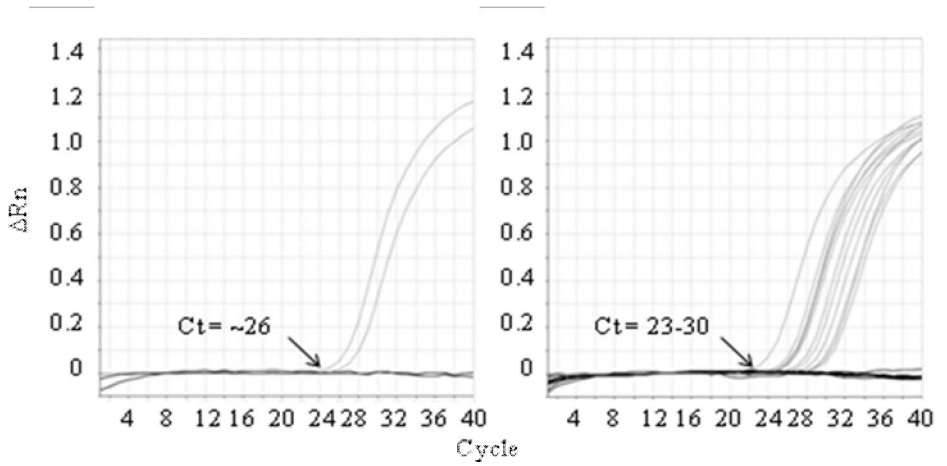

Figure 3.14: A comparison of the amplification plots and S9 C_t values produced by replicates of CaSki cells (left) and a group of HPV-16 positive patient samples (right). The grey curves were produced by S9 amplification. The black curves were produced by E6/E7 amplification.

Another group of HPV-16 positive, patient specimens were chosen based on cytology results to represent 5 different cytological categories: NIL, ASCUS, ASC-H, LSIL and HSIL; with the desire to identify a quantifiable association between the amount of E6/E7 mRNA present and the degree of cytology (Figure 3.15A-E). All samples were considered adequate for analysis, based on S9 amplification (data not shown). The NIL sample had no E6/E7 amplification (Figure 3.15A), the ASCUS sample showed E6/E7 amplification at a C_t of 31 (Figure 3.15B), the LSIL sample had no amplification (Figure 3.15C), the ASC-H sample showed E6/E7 amplification at a C_t of 28 (Figure 3.15D), and the HSIL showed amplification at a C_t of 35 (Figure 3.15E). Their analogous no RT controls showed E6/E7 amplification at C_t's of approximately 38, 36, 33 and 36 (Figure 3.15B through E, amplification plots on the right). The no RT control of the NIL specimen did not show any amplification (Figure 15A). Table 3.3 outlines the number of copies of E6/E7 mRNA, extrapolated from the standard curve, found in each sample and analogous no RT control. No association was identified between cytology grade and the amount of E6/E7 mRNA present in the sample. It is interesting to note that at very low E6/E7 copy numbers, the no RT controls (LSIL and HSIL) had a greater amount of E6/E7 than its corresponding cDNA.

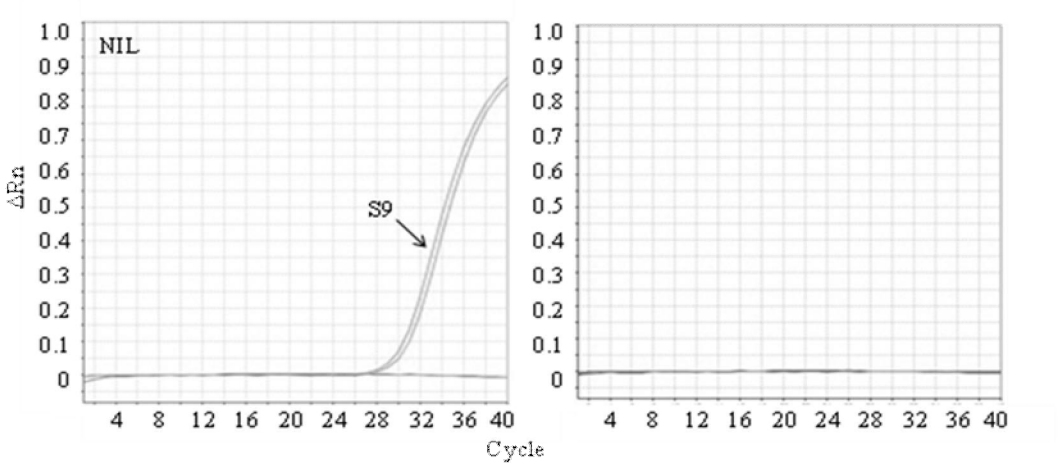

Figure 3.15A: Amplification plots produced by a HPV-16 positive, NIL patient specimen. The plot on the left corresponds to the sample, and the plot on the right corresponds to the no RT control.

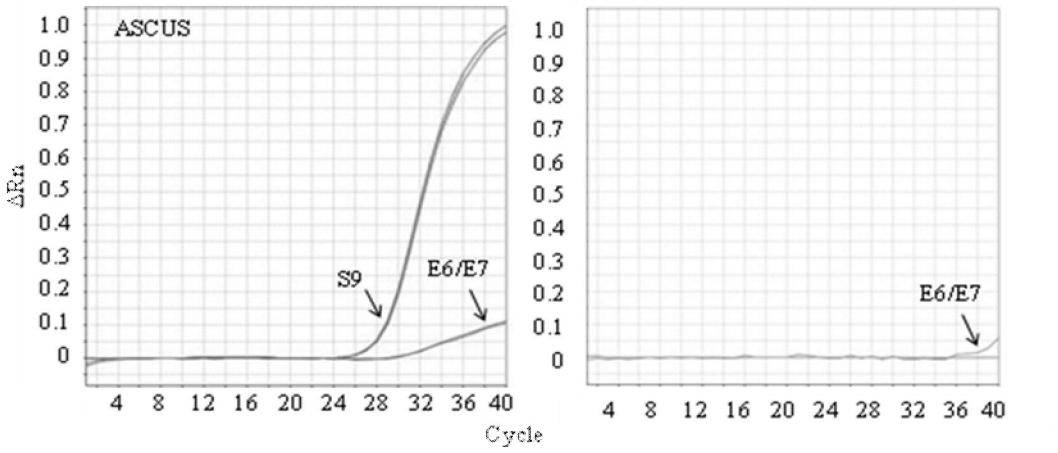

Figure 3.15B: Amplification plots produced by a HPV-16 positive, ASCUS patient specimen. The plot on the left corresponds to the sample, and the plot on the right corresponds to the no RT control.

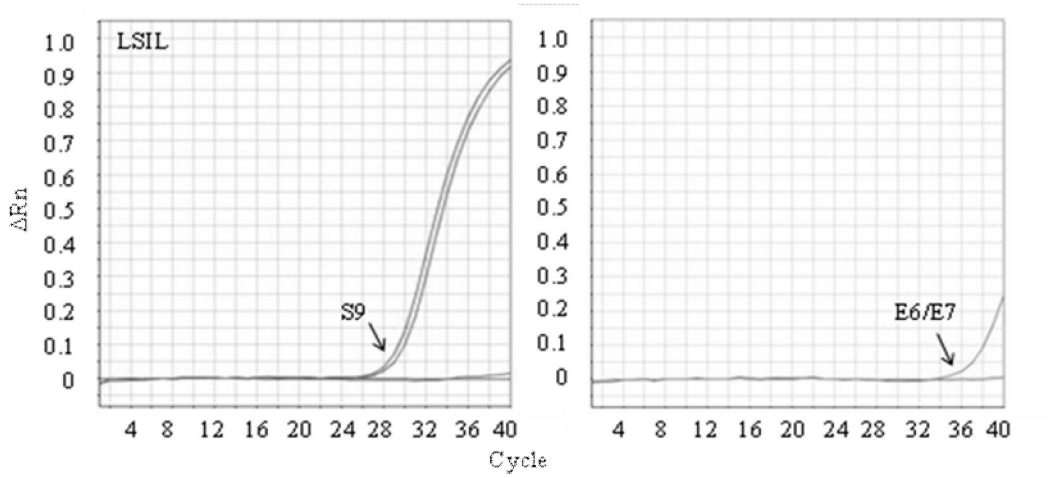

Figure 3.15C: Amplification plots produced by a HPV-16 positive, LSIL patient specimen. The plot on the left corresponds to the sample, and the plot on the right corresponds to the no RT control.

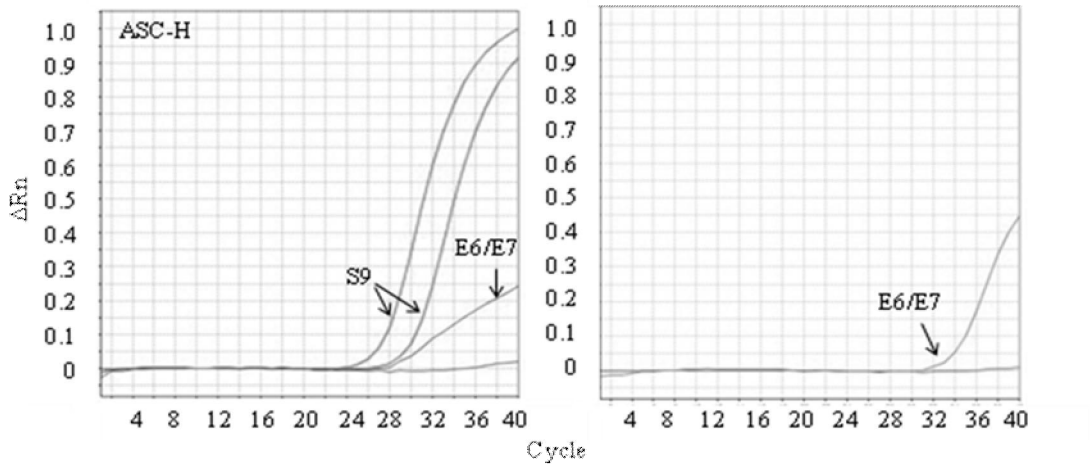

Figure 3.15D: Amplification plots produced by a HPV-16 positive, ASCH patient specimen. The plot on the left corresponds to the sample, and the plot on the right corresponds to the no RT control.

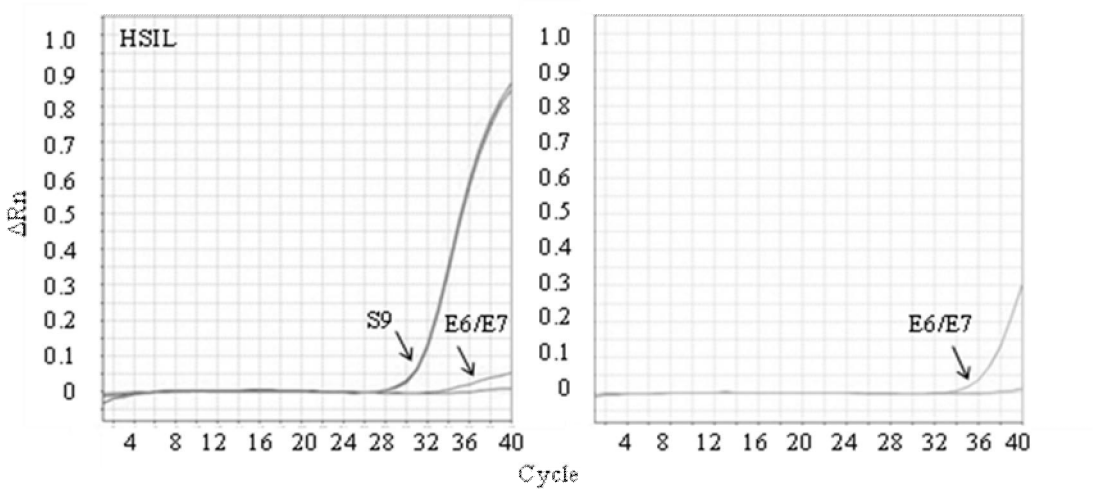

Figure 3.15E: Amplification plots produced by a HPV-16 positive, HSIL patient specimen. The plot on the left corresponds to the sample, and the plot on the right corresponds to the no RT control.

Table 3.3: A comparison of sample and no RT control E6/E7 copy numbers (copies/µL) among patients with different cytology.

Cytology	Sample Copy Number	No RT Control Copy Number
NIL	0	0
ASCUS	65	<1*
LSIL	0	3
ASCH	600	30
HSIL	3	4

*<1 copy/µL as extrapolated from the standard curve, however this is outside the range of the standard dilution series and may be considered negligible.

3.3.5 Limit of Detection

Cell mixing experiments were completed to determine the minimal amount of E6/E7 mRNA required in a sample to produce amplification via real-time PCR. The preserved control cell lines (CaSki and C33-A) were combined for a total concentration of 10^6 cells/mL and aliquoted into mixes containing 100%, 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, and 0% CaSki cells. All of the aliquots show amplification of S9, but none of them showed amplification of E6/E7 mRNA (Figure 3.16).

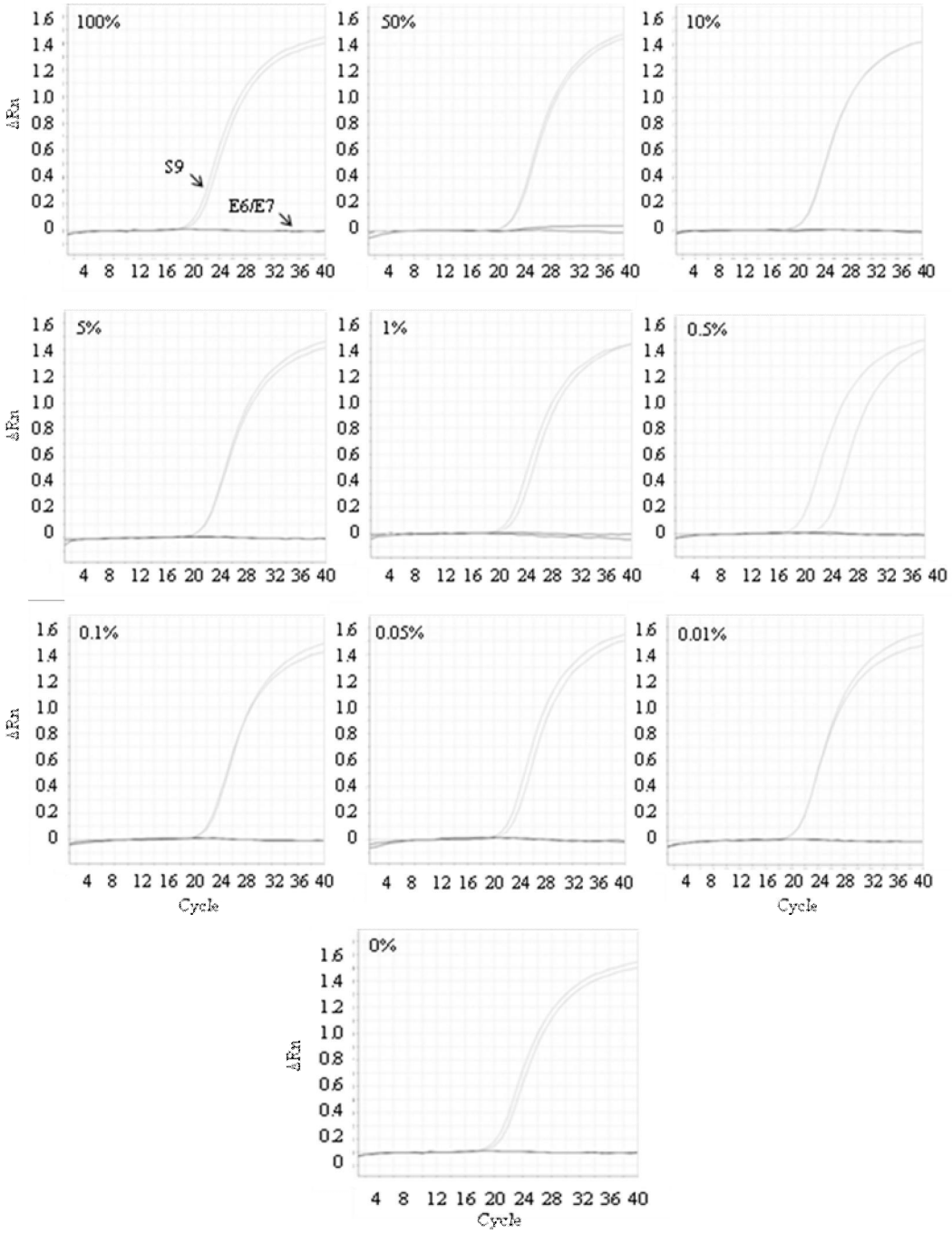

Figure 3.16: A comparison of amplification plots produced by dilutions of CaSki cells. The percent in the top left hand corner of each graph indicates the proportion of CaSki cells within the sample. The grey curves are produced by S9 amplification (as marked on the 100% plot). The black lines correspond to E6/E7 amplification (as marked on the 100% plot).

3.3.6 Normalization

HPV positive and negative controls were analyzed using fluorescent *in situ* hybridization (FISH) and flow cytometry to detect cells which were HPV positive and expressing E6/E7 mRNA. Unstained HPV controls were analyzed to determine their auto-fluorescence, and then compared with FITC-labelled HPV controls to determine if a fluorescent shift occurred; these are reported as median fluorescent intensities (MFI). A small shift in the MFIs was observed between stained and unstained CaSki and C33-A cells; however the shift should not have been the same for both controls (Table 3.4). It would be expected that the CaSki cells would have showed a much larger MFI when stained than the C33-A cells because all of the cells are HPV-16 positive and expressing E6/E7 mRNA.

Table 3.4: The MFI of each control cell population unstained and stained with FITC-labelled oligonucleotide probes

Sample	Unstained (MFI)	Stained (MFI)	Difference*
CaSki	4.74	5.78	1.04
C33-A	2.69	3.28	0.59

* Difference is calculated as the stained MFI minus the unstained MFI.

3.4 DISCUSSION

The main objective of this study was to characterize a quantifiable association of E6 and E7 mRNA expression with the presence of cervical dysplasia. A direct link has been established between the amount of E6 and E7 transcript present and the presence of varying degrees of abnormal cytology (3). Quantification of these transcripts would allow confirmation of specific cervical abnormalities, and monitored over time could predict disease progression or regression in abnormal cases of undetermined significance. Exfoliated cervical cell samples were collected in

liquid based preservative and analyzed to determine the presence of infecting HPV genotypes (Chapter 2); once genotyped, HPV-16 positive samples were subject to a total RNA extraction, reverse transcription and real-time PCR to determine the amount of E6/E7 mRNA present. The absolute quantity of E6/E7 mRNA was determined by extrapolation of the copy number from a standard curve and normalized for the proportion of cells expressing the transcript.

Previous studies have been able to amplify and/or quantify E6 or E7 mRNA in clinical samples through a variety of procedures (3, 9, 13-17); the majority of these detected the presence of either E6 or E7 of a specific genotype (3, 9, 13, 15-17). Some have also been able to correlate these findings with cytological results (3, 9, 14-16). The goal of this study, however, was to determine the absolute quantity of both gene products simultaneously and correlate this with cytological abnormality. An mRNA sequence that was conserved and required in the translation of both products was thus chosen as the target (6). Experimentation revealed difficulties in the amplification of E6/E7 mRNA from the patient samples and HPV positive controls. Samples, considered adequate for analysis based on S9 amplification, were analyzed but very limited amplification of E6/E7 mRNA was seen, and no direct associations with cytology could be made.

FISH and flow cytometry was used to determine the proportion of cells within the cervical sample expressing E6/E7 mRNA. This proportion would then be used to normalize the amount of E6/E7 transcript to only those cells expressing it. CaSki and C33-A cells were analyzed but there were no differences detected between them. All of the CaSki cells would be expected to be expressing E6/E7 mRNA, therefore when stained with FITC-labelled oligonucleotides a large shift in the MFI would be expected. In contrast, none of the C33-A cells would be expected to express E6/E7 mRNA and little to no shift in the MFI should be seen. A previous study has used this technique to detect high levels (>200 copies/cell) of E6/E7 mRNA within patient cervical samples of varying abnormalities (11). While another has been able to differentiate the proportion of ectocervical cells expressing E6/E7 mRNA among women with normal and high-grade cytology (18). This technique thus should have been acceptable for discriminating the proportion of cells expressing E6/E7 mRNA from the rest.

A main concern brought forth in this study was the inability to identify E6/E7 mRNA within confirmed HPV-16 positive patient samples and controls using either real-time PCR or fluorescent *in situ* hybridization. Early on in real-time PCR analysis, E6/E7 amplification was seen in CaSki cells, HPV-16 positive patient samples, and the standard dilution series but over time this amplification diminished. While in FISH and flow cytometric analysis E6/E7 mRNA transcripts were never detected. In the absence of changes to the primer pairs used in real-time PCR, alterations of the molecular steps throughout the quantification assay, or new control cell lines for flow cytometric analysis a possible explanation of this result could include degradation of the RNA within the liquid based preservative. Many studies have determined that HPV E6 or E7 mRNA is stable and detectable in PreservCyt® after storage at 4 °C for 14 days, 1 month and even up to 1 year (19-21). The manufacturers of PreservCyt®, however indicates that samples can be stored at ambient temperature (4 to 37 °C) for up to 6 weeks prior to analysis (22). Samples for this study were stored at room temperature in accordance with the manufacturers' suggestions, however, storage at 4 °C may have extended the time that E6/E7 mRNA would have been stable and detectable within the samples and controls; this explanation however does not account for the presence of human S9 ribosomal RNA in the samples. In order to fully analyze the quality of the RNA within a given sample one could use gel electrophoresis or Agilent's RNA Lab Chip to check for the degradation of rRNA (12). Quality assessment of the rRNA would show the extent of RNA degradation within each sample and determine whether or not the sample is suitable for analysis. Perhaps in this study viral mRNA was fully degraded while S9 rRNA was only slightly degraded allowing for its amplification to occur.

Sub-optimal primer design in the real-time PCR assay may also explain the lack of E6/E7 amplification, while S9 amplification continued to occur. As stated previously, the primer pair designed for S9 amplification has been previously published and verified by the research of others, while the primer pair for the simultaneous detection of E6 and E7 mRNA was designed specifically for this assay (3, 23). The performance of this assay would benefit from the substitution of primers with those that span introgenic sequences to specifically amplify mRNA.

The performance would also improve by the replacement of the original primers with separate primer pairs for both E6 and E7 mRNA. Both pairs should span intron/exon junctions and two distinct probes should be designed to allow for the detection of each product separately. This design is supported by the literature whereby RT-PCR was used to detect E6 from multiple genotypes using consensus primers which spanned introgenic sequences (15).

Replacement of the primers would also address the problem of amplification in the no RT controls. A late-amplification product (C_t>35) was consistently observed in the no RT negative control reactions (i.e. RNA template, not DNA) derived from RNA extractions of HPV-16 positive clinical specimens and CaSki cells. Initially, this suggested the possibility that residual DNA remained following the RNA extraction process and was causing false positive results, albeit associated to HPV only and not human DNA, as indicated by a complete absence of S9 detection. Despite varied set-up conditions for the no RT controls, including extended DNase treatments, the non-specific amplicon could not be controlled. Replacement of the primers with those spanning introgenic sequences would exclude products containing introns (i.e. contaminating DNA) and allow specific amplification of products containing exons (i.e. mRNA).

Complete characterization of this non-specific amplicon was secondary to the analysis of this study, albeit attempts were made to distinguish it from DNA probe degradation and probe dimer formation. Amplification product(s) from clinical samples, control cell lines, and no RT controls were resolved through gel electrophoresis. As expected, ~85 bp and ~120 bp products corresponded to S9 and E6/E7 in the appropriate samples and controls (Figure 3.11). In contrast, a >120 bp product was associated with the amplification observed in the no RT controls for the E6/E7 probe. The presence of this band ruled out E6/E7 probe dimerization (~50 bps) and probe degradation. Seeing as the band appeared to contain the desired target sequence, based on its approximate size and primer and probe binding, this would suggest that there was contaminating DNA in the RNA extracts which contains some sort of post-transcriptional modifications to make them larger (12, 24, 25).

The performance of the quantification assay would also benefit through the modification of primers used in the reverse transcription step. Reverse transcription in this assay was completed using random primers. Random primers were chosen because they do not require high quality RNA template, produce the least bias and the largest amount of cDNA (12). Disadvantages of this strategy include over-priming and a lack of reliability of priming targets present at low levels (12). In comparison, oligo-dT primers require the presence of a polyA tail and a high quality RNA template (12). They further differ from random primers because they do not allow for the reverse transcription of rRNA, which is typically in excess in total RNA extractions. Although previous reports suggest that random primers allowed for better detection of HPV-16 E6/E7 oncogenic transcripts than oligo-dT primers due to the low quality of RNA in the samples, this priming strategy may be more effective (21). In the presence of high quality RNA, the real-time PCR reaction could continue to be multiplexed with the use of oligo-dT primers. This approach would not only specifically reverse transcribe mRNA, but it would consequently decrease the amount of unrelated template entering the PCR reaction and problems associated with this. Seeing as the amount of HPV mRNA in the clinical samples is very low, reproducible detection of these would be expectantly low due to the stochastic sampling effects (24). The Monte Carlo effect outlines that the true abundance of low abundance transcripts are less likely to be represented accurately because of the low probability of primer annealing (12). The use of oligo-dT primers could potentially mitigate these effects and increase the specificity of both the reverse transcription and PCR reactions.

Lastly, fluorescent *in situ* hybridization and flow cytometric analysis did not identify E6/E7 mRNA expression within CaSki or C33-A cells. One possible explanation for this includes the autofluorescence of these cell lines which can be defined as the fluorescence emitted by the cells in the absence of a fluorochrome or dye. The main concern with autofluorescence is that it makes it difficult to detect the emissions from fluorescently labelled probes when the autofluorescence is emitting in the same spectrum. Flow cytometric analysis of unstained cells would reveal their autofluorescence so that one could correct for this when stained cells are

analyzed; but if the autofluorescence blocks detection of the probe fluorescence when the cells are stained no change will be detected. E6/E7 expression in CaSki cells may therefore be difficult to detect if the autofluorescence of the cells and the FITC emissions cannot be differentiated. If this is the case, it would be necessary to identify control cell lines that have less autofluorescence or a different emission spectrum.

3.5 CONCLUSION

The assay developed in this study at present cannot be used to determine the absolute quantity of E6/E7 mRNA with in cervical cell samples. Methodologically, changes need to be made in primer design, the reverse transcription priming strategy, the control cells being used in flow cytometry, and the quality of the RNA template being used in quantification. Further research is required to fully characterize the degradation of the RNA within the liquid based preservative to determine its feasibility for molecular analysis. Also, analysis is required to identify the optimal storage conditions for samples within the liquid based preservative to prevent, or minimize the degradation from occurring. Future directions in this study should include longitudinal analysis of patient samples with abnormal cases of cervical dysplasia of undetermined significance. Quantification of these oncogenic viral transcripts throughout the follow-up period may provide prognostic indicators important in the triage of these cases.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 THESIS OBJECTIVES

The main objective of this study was to characterize a quantifiable association between HPV E6 and E7 mRNA expression and the stages of cervical disease. Quantification of these oncoproteins are said to be better predictors of cervical disease than current diagnostic markers (1). Quantification of these markers was attempted through the collection of exfoliated cervical cell samples into a liquid based preservative, followed by the detection and genotyping of infecting HPV types, and quantitative, multiplex real-time reverse transcription PCR.

4.2 SUMMARY OF RESULTS

Exfoliated cervical cell samples were collected from a colposcopy-based population, representing a group of women that are at a higher-risk of developing cervical carcinoma due to pre-existing cervical abnormalities. This study group was chosen because associations between particular high-risk HPV types and specific stages of cervical disease could be drawn and regional HPV genotype prevalence and distribution could be predicted; the assertion of this prevalence is key in monitoring the effectiveness Gardasil®, which currently only protects against HPV-16, HPV-18, HPV-6, and HPV-11. Genotyping of the sample population revealed that the most prevalent types were HPV-16, HPV-31, HPV-52, and HPV-39, -42, and-56. This study confirmed that HPV-16 is one of the most prevalent types, followed by other high-risk HPV types. In agreement with other Canadian and international genotype prevalence studies, HPV-18 was not found to be among the most prevalent types (2-13). Also, variations were found among high-risk HPV types and their associations with different grades of cervical disease, suggesting that not all high-risk HPV types play an equal role in the development of cervical cancer (2). Although, the sample population lacks generalizability it does provide a good aspect of the high-risk HPV types

currently present in the community that may be associated with cervical disease and should be targeted by the vaccine in the future.

Once genotyped, HPV-16 positive patient samples were analyzed to determine the absolute quantity of HPV E6/E7 transcript present, as a direct link has been established between the amount of transcript present and the presence of varying degrees of abnormal cytology (14-17). Quantification of these transcripts thus allows for the confirmation of specific cervical abnormalities, and longitudinally could predict clinical significance in abnormal, or low-grade, cases of undetermined significance. The simultaneous absolute quantification of both viral oncogene transcripts was attempted using a multiplex real-time reverse transcription PCR assay coupled with a normalization procedure involving fluorescent *in situ* hybridization and flow cytometric analysis, to limit the quantity of E6 and E7 to the proportion of cells actively expressing them. But due to the surprisingly sub-optimal performance of this assay, E6 and E7 transcripts could not be quantified. Potential reasons for this include degradation of HPV RNA in the liquid based preservative, inferior design of PCR primers, poor choice of reverse transcription primers, and the absence of proper controls in the normalization assay. All of the aforementioned inferiorities are potentially reparable in the re-designing of this assay for future studies; samples could be stored at 4 °C instead of room temperature, individual PCR primers could be designed for each viral transcript, oligo-dT primers could be used for reverse transcription, and a control cell line with well characterized auto-fluorescence could be used in sample normalization (18-21). With regards to RNA degradation, further research is still required to fully characterize the extent of degradation within a sample stored in liquid based preservative under various storage conditions, to ensure suitability of the samples for molecular analysis.

4.3 RELEVANCE

Infections with high-risk HPV types are significant in the development of cervical pre-cancers and cancers and the ability to detect these infections would have a large impact on cervical cancer screening programmes. Recent molecular advancements and technologies in the field of

HPV detection and quantification have the ability to transform these programmes and further decrease the incidence and mortality associated with cervical cancer that is currently limited by the Pap smear. These molecular diagnostic tests, designed to target markers of active HPV infections, will eventually allow for the detection of HPV and confirmation of the presence of clinically significant disease, prior to development of cervical cancer.

4.4 FUTURE DIRECTIONS

Although out of the scope and time limit of the current study, it would be interesting to observe the longitudinal analysis of patient samples with varying degrees of cytology using the above methodology. This analysis would provide information about the HPV genotypes that appear to persist throughout follow-up, and the changes in viral transcript quantities over time and how these correlate with changes in cervical disease. Ultimately, follow-up analysis would allow for the characterization, identification and triage of patients with progressive HPV infections and cervical disease that are clinically significant and require increased surveillance.

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