THE UTILITY OF HPV TYPING AND RELATIVE QUANTIFICATION OF HPV-16 TRANSCRIPTS FOR MONITORING HPV VACCINE EFFICACY AND IMPROVING COLPOSCOPY TRIAGE OF WOMEN WITH ABNORMAL CERVICAL CYTOLOGY

A Thesis
Submitted to the Faculty of Graduate Studies and Research
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biochemistry
University of Regina

By
Nick Anthony Antonishyn
Regina, Saskatchewan
February 2008

Your file Votre référence
ISBN: 978-0-494-42500-8
Our file Notre référence
ISBN: 978-0-494-42500-8

UNIVERSITY OF REGINA

FACULTY OF GRADUATE STUDIES AND RESEARCH

SUPERVISORY AND EXAMINING COMMITTEE

Nick Anthony Antonishyn, candidate for the degree of Doctor of Philosophy in Biochemistry, has presented a thesis titled, ***The Utility of HPV and Relative Quantification of HPV-16 Transcripts for Monitoring HPV Vaccine Efficacy and Improving Colposcopy Triage of Women with Abnormal Cervical Cytology,*** in an oral examination held on February 8, 2008. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

External Examiner:	*Dr. Martin Petric, University of British Columbia
Co-Supervisor:	Dr. Rod A. Kelln, Department of Chemistry and Biochemistry
Co-Supervisor	Dr. Greg Horsman, Adjunct Professor, Department of Biology
Committee Member:	Dr. Andrew Freywald, Department of Chemistry and Biochemistry
Committee Member:	Dr. Dae-Yeon Suh, Department of Chemistry and Biochemistry
Committee Member:	Dr. Richard Manzon, Department of Biology
Committee Member:	Dr. Alberto Severini, National Microbiology Lab
Chair of Defense:	Dr. Dongyan R. Blachford, Faculty of Graduate Studies and Research

*External in Absentia

ABSTRACT

Human papillomaviruses (HPV) can cause benign or malignant disease with the majority of infections without symptoms. The viral etiology of cervical cancer is now proven with HPV proteins E6 and E7 defining part of the molecular basis of oncogenesis *in vitro*. Integration into chromosomes and/or malignant transformation of cervical cells *in vivo* are expected to be accompanied by the over-expression of HPV genes for E6 and E7 oncoproteins and a reduction of expression for the L1 capsid protein and replication of viral DNA. Cervical intraepithelial neoplasia (CIN) has been associated with particular HPV types that can be distinguished by DNA sequence differences. The research work studied two important aspects of HPV and its role in cervical disease. First, the distribution of HPV types and the epidemiology of HPV infection in a population of Saskatchewan women referred to a colposcopy clinic. Second, the potential of HPV-16 transcripts and HPV viral load for the detection of CIN.

The most commonly identified HPV genotype in patients with CIN grade 2 or worse was HPV-16 (46.7%) followed by HPV-31 (14.7%) and then HPV-18 (3.9%). The risk of CIN associated with HPV-18 infection, odds ratio 0.8 (95% CI, 0.4 to 1.7) is significantly lower than either the odds ratio of 6.3 for HPV-16 (95% CI, 3.6 to 11.0) or 4.3 for HPV-31 (95% CI, 1.8 to 12.6). Thus in Saskatchewan, the prevalence of HPV-31 is high whereas HPV-18 is associated with less clinical disease. Consequently, the efficacy the recently introduced cervical cancer vaccine, which targets only the oncogenic types HPV-16 and HPV-18, may be diminished in Saskatchewan's population.

Analysis of variance (P = 0.2) indicated no significant correlation between grade of CIN and HPV viral load. The presence of E6 RNA (P = 0.0002) and relative quantification of HPV-16 E6 transcripts (P < 0.0001) displayed the most significant median difference among the various grades of CIN when standardized to HPV viral load and human RNA and DNA levels. There was no correlation with L1 transcripts and cervical disease. Likelihood ratios indicate that the combination of Pap smear cervical cytology screening test with E6 relative quantification, on populations with higher cervical disease prevalence, would identify more true positive cases than simply an additional Pap test. Using molecular testing for triage, HPV genotype information identifies 96% of women with CIN grade 2 or worse while eliminating 44% of women with CIN grade 1 or better. Information from the relative quantification of HPV-16 E6 transcripts identified 31.0% (n=13) of HPV-16 positive women with CIN grade 1 or better while retaining 92.4% of women with CIN grade 2 or worse.

This work shows that there is diagnostic utility in relative quantification of HPV transcripts and that it benefits from standardization for variables such as the amount of HPV DNA and the total cellular nucleic acids. Relative quantification of HPV-16 E6 and HPV genotyping can be used to reduce medical procedures for women. HPV molecular tests could be useful in a cascade of diagnostic testing designed to refer women with cervical abnormalities for colposcopy, or treatment, while reducing the number of women needing triage.

ACKNOWLEDGEMENTS

I'd like to express my gratitude to Dr. Dennis Lehotay for giving me the incentive to purse my doctoral degree in the first place. Of course, this work would not have been possible without the scientific ideas, financial support, and practical advice of my immediate advisory committee consisting of, in alphabetical order, Dr. Greg Horsman, Dr. Rod Kelln and Dr. Alberto Severini. Dr. Betty Brydon and Joanne Guillemin were absolutely invaluable in the acquisition of clinical material. Specials thanks for participation of the following physicians: L.A. Brydon, R. Bhargava, J. Bastian, D. Shepherd, A. Akinbiyi, R. Cardoso and C. Jabs. Many thanks to Brad Cargill and Joyce Kirsch for pulling together the Pap and histology data. This research was partially funded through an NSERC grant to RAK. The Saskatchewan Disease Control Laboratory fully supported all aspects of the degree and research and for this I'm personally very grateful.

DEDICATION

This work and all that I do is dedicated to Deanne, Isis and our brand new baby girl. Isis gave me the means and motivation to finish while Dee made it all possible with her sacrifice and support. Thanks Dee for doing everything else so that I could do this!

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LIST OF ABBREVIATIONS

AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
ASCUS	Atypical squamous cells of undetermined significance
ATCC	American Type Culture Collection
BHQ	Black Hole Quencher®
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cdks	Cyclin-dependent kinases
CIN	Cervical intraepithelial neoplasia (either grade 1, 2 or 3)
CIS	Carcinoma in situ
Ct	Cycle threshold
Da	Dalton
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA Lab	Molecular Diagnostics Section, Provincial Laboratory
DNA	Deoxyribonucleic acid
DOB	Date of birth
dTTP	Thymidine 5'-triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetic acid

ELISA	Enzyme-linked immunosorbant assay
EV	Epidermodysplasia verruciformis
FAM	6-carboxyfluorescein
HBV	Hepatitis B virus
HEPES	*N*-[2-Hydroxyethyl] piperazine-*N*'-[2-ethanesulfonic acid]
HSIL	High-grade squamous intraepithelial lesion
HPLC	High pressure liquid chromatography
HPV	Human papillomavirus
HSN	Health services number
HTLV-I	Human T-cell lymphotropic virus type I
ICC	Invasive cervical cancer
KOD	Kinetic outlier detection
LCR	Long control region
LEEP	Loop electrosurgical excision procedure
LSIL	Low-grade squamous intraepithelial lesion
LR	Likelihood ratio
MHC	Major histocompatibility complex
NPV	Negative predictive value
Oligo	Oligonucleotide
ORFs	Open reading frames
Pap	Papanicolaou smear
PCR	Polymerase chain reaction
PPV	Positive predictive value

QC	Quality control
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
RQ	Relative quantification
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SIL	Squamous intraepithelial lesion
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
URR	Upstream regulatory region
VLPs	Virus-like particles

1. INTRODUCTION

1.1 Epidemiology of cancer

For 2006, it was expected that there would be approximately 150,000 new cases of cancer diagnosed in Canada resulting in 70,000 deaths. It is expected that 38% of women and 44% of men in Canada will be diagnosed with cancer at some point in their lifetime and almost one of every four Canadians will die of cancer. Of the above cancers, it was estimated that there would be 1,350 new cases of cervical cancer and 390 cervical cancer-related deaths. Cervical cancer is the eleventh most common cancer diagnosis and the thirtieth most common cancer-related cause of death in Canadian women (Canadian Cancer Society, 2006 Annual Report). The United States National Cancer Institute's histology program recorded 162,769 female genital tract cancers over a 15-year period (1973-1987) and found cervical cancer at 20% to be the third most common, preceded by endometrial cancer at 48% and ovarian cancer at 26% (Platz & Benda, 1995). This same study found that squamous cell carcinoma accounted for 77.1% of cervical cancers followed by adenocarcinoma at 10.9%.

In Saskatchewan, the number of new cancers in females has increased by 47.7% over the past two decades, with an average annual percent increase of 1.4 %. The overall increase in age-adjusted incidence of cancer for women from 1983 to 2002 was 20% (Tonita & Alvi, 2004). In Saskatchewan men, the age-adjusted increase in incidence was only 13% for the same period. In 2002, the number of deaths in Saskatchewan women due to cancer was 965, compared to 693 in 1983. Likewise, the number of new invasive cancer diagnoses in

Saskatchewan females was 2,140 in 2002, compared to 1,449 in 1983. On the contrary, the overall incidence of cervical cancer has declined in Canada during the same period, mostly due to the institution of Pap screening, with a drop in the annual incidence rate from 15 to 10 per 100,000 women (Franco et al., 2001). The annual incidence for cervical cancer for Saskatchewan women in 2000 was 9.5 per 100,000, which is slightly below the national average of 9.7 (range 8.3 to 13.0). However, the incidence of cervical cancer is highest among Aboriginal Canadians in Saskatchewan (representing 29% of all cancers within Aboriginal women compared to 1.8% for the national average), with an age-standardized incidence six times higher than the national average (Franco et al., 2001). This figure exceeds the highest reported incidence for a defined group, which was 44.3 per 100,000 in East Africa (Haverkos, 2005).

1.2 Viruses associated with human cancer

As the oncogenic properties of many viruses became documented in animals, it was assumed that some human malignancies were also virus-induced. At first, the evidence for viral oncogenicity in humans was indirect. The Epstein-Barr virus (EBV) is a common infectious agent and the cause of mononucleosis. It was the first virus linked to human cancer through seroepidemiologic studies whereby cancer was a rare outcome of a common infection (Klein et al., 1969). While serological studies have linked EBV with oncogenicity in humans, the strongest evidence came with the consistent identification of the virus in tumour cells. EBV is regularly detected in tumour specimens from patients with Burkitt's lymphomas and nasopharyngeal

carcinoma (Longnecker, 1998). Its ubiquity has proven that the presence of infection is not a reliable predictor of malignancy.

Tumour-association was also important in establishing the link of human papillomavirus (HPV) with cervical cancer. Most oncogenic viruses elicit the production of lifelong antibodies that are readily detected in healthy and diseased individuals, e.g., EBV, human T-cell lymphotropic virus type I (HTLV-I), and the hepatitis viruses (Tanaka et al., 2006; Wang et al., 2005). In contrast, antibodies to HPV is not readily detected in cancer cases (Wang et al., 2003). Consequently, its association with human cancer has required the advent of DNA hybridization technology, since useful serological assays were not available at the time (Zachow et al., 1982).

Today, there are five groups of viruses with well-documented links to human cancer; they are: the herpes viruses (particularly EBV and HHV-8), the hepatitis viruses (HBV, HCV), the retroviruses (HTLV-I), the polyomaviruses (JC, BK and SV40 viruses) and the human papillomaviruses (HPV). Both polyomaviruses and papillomaviruses once belonged to a now defunct family known as 'Papovaviridae' since both groups comprise small viruses (<60 nm) with a non-enveloped icosahedral capsid and a genome made up of double-stranded circular DNA organized into functional regions. Their growth cycles are slow and involve replication in the nucleus. These viruses share important similarities in the manner of their gene product interactions with certain host proteins. The polyomavirus T antigen and HPV E6/E7 each interfere with the function of the human cellular tumour suppressor genes p53 and Rb. An

association between polyomaviruses and human cancers has been investigated especially since SV40 was discovered in 1960 as a contaminant of early poliovirus vaccines (Paracchini et al., 2006). Interestingly, the discovery of papillomavirus in 1933 was the origin of DNA tumour virology with the work of R.E. Shope (Shope, 1932). It was later shown by others that the Shope papillomavirus could produce malignant carcinomas in domestic rabbits (Rous & Beard, 1934). However, work with the virus became stagnant for decades due to the inability to propagate the virus in culture (Orth et al., 1978).

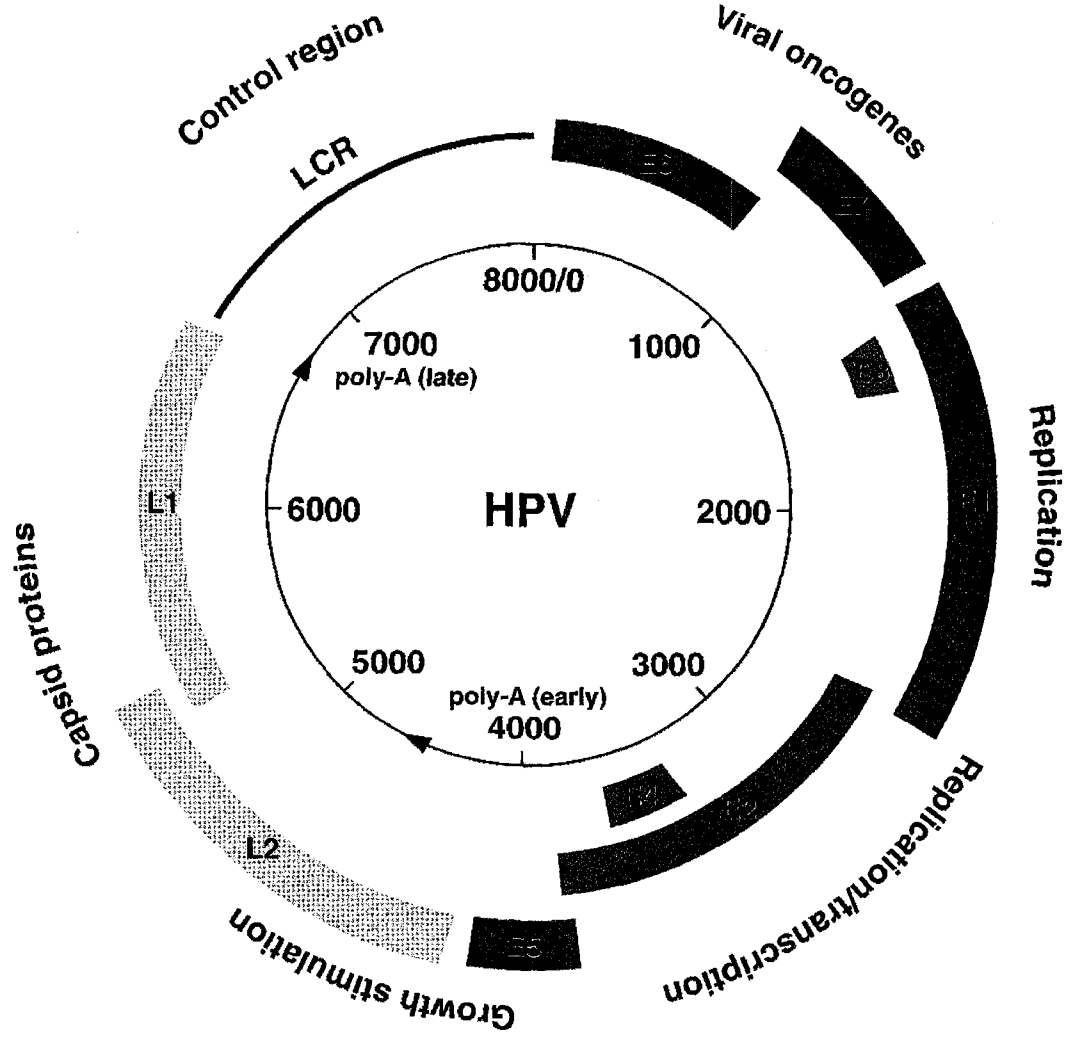


Figure 1. Genome organization of human papillomavirus.

The genetic map of HPV-16 is illustrated. Open reading frames (ORFs) are indicated with solid bars. The seven early ORFs (E1, E2, E4, E5, E6, E7 and E8) are expressed at different stages of epithelial differentiation. L1 and L2 ORFs are expressed in cells replicating viral DNA in upper differentiated epithelial cells. Taken with permission from (Prendivillie, 2004). Original in color.

1.3 Papillomaviruses

The name papillomavirus developed from the Latin term 'papilla', meaning nipple or pustule, and the Greek suffix '–oma', which means tumour (Sanclemente & Gill, 2002). Papillomaviruses are specific for their respective hosts and are named accordingly e.g., Cottontail rabbit papillomavirus (CRPV - originally called Shope papillomavirus). In 2004, the International Committee on Taxonomy of Viruses officially recognized papillomaviruses as a separate family apart from polyomaviruses, which together formed the now defunct Papovaviridae family (de Villiers et al., 2004). The two families of viruses are now separated since they have different genome sizes, different genomic organizations and no recognizable nucleotide or amino acid sequence similarities (de Villiers et al., 2004). The family Papillomaviridae contains a highly diverse collection of over 180 viruses that have suitable phylogenetic criterion for taxonomic classifications, which include genus, species, types, subtypes and variants.

All papillomaviruses have circular double-stranded DNA genomes that are approximately 8 kb in size. Protein-encoding sequences are found on only one DNA strand and the open reading frames (ORFs) are designated 'early' or 'late' to indicate their time-line of expression in the normal viral replication cycle (Figure 1). Eight early ORFs and two late ORFs have been identified to date but not all types possess all ORFs.

Overlapping ORFs have been found in the genomes of all HPV types (Severson et al., 2001). The early genome region is transcribed into partially

overlapping coding regions that are differentially spliced and share a common 3' end at the beginning of L2, which is defined by a polyadenylation signal (Seedorf et al., 1985). Most types have two regulatory proteins, E1 and E2 that modulate the transcription and replication and three oncogenes, E5, E6 and E7 that modulate the transformation process, and two structural proteins, L1 and L2 that form the viral capsid.

Changes by mutation or recombination events are very rare within papillomaviruses genomes and are seen at frequencies similar to the genomes of the infected host organism, estimated at 1% nucleotide exchange per 100,000 to 1,000,000 years (de Villiers et al., 2004). The L1 gene is the most conserved and is therefore used for identification of papillomavirus types (van den Brule et al., 1990). A 291 bp segment of the L1 gene can be used for typing since it has been shown to suffice as a foundation for highly informative phylogenetic comparisons (Bernard et al., 1994). The complete L1 gene is required for definitive phylogeny and taxonomy of papillomaviruses and can be used for the identification of new HPV types (de Villiers et al., 2004).

The genera of papillomavirus share less than 60% nucleotide sequence identity in the L1 gene (de Villiers et al., 2004). Greek letters were introduced to name the genera (e.g., all genital HPV are alpha-papillomaviruses). Species share between 60% and 70% nucleotide identity and are identified by a numbering system. The term "type species" has been coined for the best-studied type within a species (de Villiers et al., 2004). Traditionally, a new 'type' is designated if the L1 ORF differs by more than 10% from the closest known

papillomavirus type. This has been refined in the new classification system to encompass all papillomaviruses that share between 71% and 89% nucleotide identity. A numbering system is used to identify a type, for example HPV-1, an abbreviation of human papillomavirus type 1. Differences between 2% and 10% similarity define a subtype and less than 2% a variant (Bernard, 2006). Very few subtypes or variants have been found, which is speculated to be due to slow and linked evolution of host and virus (Chen et al., 2005; de Villiers et al., 2004).

1.4 Human papillomaviruses

It is speculated that humankind has always been infected by papillomaviruses given the low rate of change of the papillomavirus genome and because all ethnic groups across the world contain the same 'set' of high-risk genital HPV types (Bernard, 2006). HPV is the most common of the sexually transmitted infections in most populations (Burd, 2003). Estimations of prevalence of genital HPV DNA ranges from 7 to 37% depending on the country and the age of the study group (Bosch et al., 2006).

Over 80 types of HPV have been identified and shown to be associated with a wide variety of benign and malignant epithelial lesions. HPVs can be classified according to their tissue tropism into dermatotropic and mucosotropic groups. However, viral genital HPVs are more commonly classified by their oncogenic potentials into low-risk and high-risk types (Severson et al., 2001). Genital HPV types are mucosotropic and have been subdivided into low-risk types, which typically produce benign genital warts, and high-risk types, which are more frequently associated with invasive cervical cancer. Epidemiologic

classification, based on odds ratios for the development of cancer, has identified fifteen high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), three probable high-risk HPV types (26, 53, and 66) and twelve low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108(Munoz et al., 2003).

There are three categories of HPV infection, based on clinical symptoms: latent, subclinical and clinical (Snijders et al., 2006b). Latent infections can only be detected with molecular tests and cause no clinical signs or cytological abnormalities. Subclinical infection can be found with colposcopy or cytological techniques. Clinical infection is associated with clinical symptoms and has visible lesions. Some HPV types produce benign lesions whereas certain types are highly associated with malignancy since, in a subset of patients, they produce lesions which can progress to *in situ* and then invasive cancer. Hyperplasia and hyperkeratosis are common pathologies of clinical infection and largely depend on the type of HPV causing the infection (Severson et al., 2001).

HPV types 1, 2 and 4 cause common warts and plantar warts. Epidermodysplasia verruciformis (EV) is an HPV skin infection with disseminated wart-like lesions that is caused by over fifteen different HPV types of which only HPV 5 and HPV 8 are commonly detected in squamous cell carcinoma lesions (Bernard, 2006). Approximately 50 mucosotropic HPV types have been reported. Of these, HPV 6 and 11 cause both oral and laryngeal papillomas while types 7, 16 and 32 are mostly associated with oral papillomas (Chang et al., 1991; Manos et al., 1999; McKaig et al., 1998). Genital infections by HPV

cmay manifest as condyloma acuminatum or veneral warts with types 6 and 11 as the most common, but these infections rarely progress to carcinoma (Severson et al., 2001). Other HPV types that infect the genital region can cause serious clinical disease, such as cervical intraepithelial neoplasia (CIN) and even cancer.

1.4.1 Epidemiology of HPV

The link between HPV and cervical cancer is now well established to point of becoming a model of viral carcinogenesis. It is generally assumed that, in practical terms, cervical cancer does not occur without the persistent presence of certain types of HPV. In fact, cervical cancer has been recognized to behave like a sexually transmitted disease long before HPV infection was implicated in its pathogenesis (Arends et al., 1998). However, it is also well-known that infection with HPV is extremely common and has a range of clinical manifestations from asymptomatic and self-limited to various stages of malignant progression that are not restricted to the cervix. Nevertheless, most cancers caused by HPV occur in the transformation zone of the cervix, where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the exocervix.

Since HPV infection is not a nationally notifable disease, its prevalence and incidence in Canada are limited to studies on select populations that have been published in the scientific literature. The overall prevalence of HPV, considering all types, ranges from 10.8 to 29.0% among populations studied and 3.4 to 42.0% among different age groups (PHAC, 2007).

Although approximately 50 HPV types can infect the genital tract, only fifteen of these are regularly found in cervical cancer and in higher proportion than controls. This subset is termed high-risk and currently is comprised of HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (Munoz et al., 2003). HPV-16 and HPV-18 have been found to be substantially more likely to progress to CIN3 and together account for 70% of cervical cancer in Canada (PHAC, 2007). The majority of world-wide cervical cancer cases are caused by HPV-16 (57%) with the second most common (17%) associated with HPV-18, and most benign infections (i.e. genital warts) were caused by HPV-6 or HPV-11 (Munoz et al., 2003). A recent prevalence study conducted in British Columbia found HPV-16 and HPV-18 in 10.6% and 3.5% respectively of women enrolled in rountine cytological screening (Moore et al., 2006). This is consistent with world-wide distribution patterns found with meta-analysis (Clifford et al., 2003).

1.5 Papillomavirus replication cycle

Papillomavirus infection can be either active or persistent. Active infection follows the viral replication cycle, whereas in persistent infection, the cycle is arrested. The productive replication cycle of HPV is linked to differentiation of the infected epidermal cells and can be divided into four phases: 1) adsorption and penetration, 2) cell proliferation and episomal maintenance in the lower epithelial layers, 3) genome amplification and 4) expression of capsid proteins. Papillomaviruses do not always go through their full productive replication cycle. A subset of benign tumours progress to carcinoma and its constitutent

transformed cells are no longer permissive for virion production. However, transformation and tumour progression are rare events.

Infection by papillomavirus requires microabrasions to expose cells in the basal layers of epithelium to viral entry (Day & Schiller, 2006). Basal layer cells provide a reservoir for suprabasal layers, and, as such, are always in a state of growth. Since these cells are continuously dividing, they possess cellular polymerases and accessory proteins that mediate DNA replication. This replication mechanism of the host cell is recruited by the papillomavirus through the E1 and E2 proteins for its own replication (Conger et al., 1999).

HPV infection of basal cells leads to the activation of viral E6 gene expression. E6 protein inhibits apoptosis and differentiation of the basal cell, which maintains episomal genomes (Song et al., 2000). This results in the production of 20 to 100 extrachromosomal copies of viral DNA per cell, which is maintained during productive infection (Day & Schiller, 2006; Sanclemente & Gill, 2002). This copy number ensures that episomal HPV DNA is present within both daughter cells after host cell division. Daughter cells will eventually detach from the basal layer and migrate to the stratum granulosum, undergoing differentiation along the way. In normal uninfected epithelia, cells exit the cell cycle once they leave the basal layer and often lose their nuclei during the differentiation process (Lippens et al., 2005). However, HPV-infected cells remain active in the cell cycle due to the action of the E7 protein of the papillomavirus (Cheng et al., 1995).

HPV proteins stimulate the proliferation of cells and this leads to increased amounts of cells originating from the basal layer. Benign HPV infection delays normal differentiation. Therefore, proliferation coupled with delayed differentiation induces spinous layer hyperplasia or the formation of wart tissue (Sanclemente & Gill, 2002). Infected suprabasal cells, during the productive life cycle, will enter the S phase in the highly differentiated state and activate the expression of the cellular replication factors required for viral replication (Longworth & Laimins, 2004b).

In this way, viral oncoproteins are necessary for cell immortalization and maintenance of the cell cycle capacity. Abrogation of cell cycle checkpoints is more effectively achieved by the high-risk HPV types (Syrjanen & Syrjanen, 1999). In certain conditions, the viral oncoproteins can become highly overexpressed by both up-regulation of the major early promoter of the virus and by increased stability of the mRNAs of these proteins (Graham, 2006; zur Hausen, 1996).

HPV oncoproteins can eliminate cell cycle checkpoints by binding to cyclin-cdk complexes and other components of the regulatory pathway (Southern & Herrington, 2000). During the normal cell cycle, the phosphorylation of Rb by G_1/S cyclins and the subsequent release of the E2F transcription factor are the triggers for the activation of the genes responsible for entry into S-phase (Middleton et al., 2003). In HPV-infected cells, their activation is regulated by viral protein E7, which binds to Rb and stimulates the release of the E2F

transcription factor, which is normally not found in uninfected epithelium (Keating et al., 2001).

However, malignant cells can arise from those HPV-infected cells in which the delicate balance of cellular takeover has failed. This leads to the loss of the cells ability to differentiate. This is devastating to the productive infection of papillomavirus because maturation of virion particles are restricted to terminally differentiated cells of the epithelial superficial layer (Fushs & Pfisher, 1997). Obviously, transformed cells are deleterious for the host since they can leads to invasive cancer, under the appropriate conditions, and ultimately host death.

1.6 Gene expression of papillomaviruses

HPV transcription is a complex process since it involves multiple promoters, differential activities of the promoters, and several mRNAs from each ORF, which are generated with alternate splicing (Severson et al., 2001). Papillomaviruses transcribe both early and late genes in a unidirectional fashion and most viral transcripts are expressed as polycistronic messages. For example, the polycistronic messages of HPV-31 have been classified into five classes (I-V) (Sherman et al., 1992). The major early transcripts (class I) contain either full-length E6 or a truncated form, which may or not be translated. Early transcripts coding E2, E5 and an E6^E4 fusion product are designated class II. Class III encodes E1 as an unspliced transcript, which may serve as a precursor for a differentiation-dependent increase in viral replication (Klumpp & Laimins, 1999). Classes II and III initate at the promoter within the E7 gene. Class IV encodes E1^E4/E5 and terminates at an early polyadenylation signal, which is at

the 3' end of E5 and immediately before L2. Class V contains E1^E4 and L1 or E5/L2/L1. Class V transcripts terminate at the late polyadenylation signal downstream of L1.

The upstream regulatory region (URR), also called the long control region (LCR), is a noncoding region of 400-850 bp, which separates the early and late gene clusters (Kalantari & Bernard, 2006). The URR contains the origin of DNA replication, promoters and other regulatory elements (Turek, 1994). Generally, genes required early in the life cycle are controlled from a promoter located within the URR while the late genes are controlled by a promoter within the E7 gene (Kalantari & Bernard, 2006). However, potential promoters and regulatory elements are continually being discovered (Braunstein et al., 1999; Sen et al., 2004; Sen et al., 2002). The E7-localized promoter is known to be differentiation dependent (Cheng et al., 1995).

Regulation of gene expression has been studied in several HPV types and each appears to have a major promoter upstream of the E6 ORF within URR. In HPV-16 and HPV-31, this promoter is called P_{97} (Hummel et al., 1992; Smotkin & Wettstein, 1986). A homologous promoter within the URR of the HPV-18 genome is called P_{105} (Thierry et al., 1987). In fact, all alpha-papillomaviruses have analogous promoters within the URR (Kalantari & Bernard, 2006).

P_{97} is the major early promoter directing all expression of the ORFs during the early phase of the viral life cycle (Rosenstierne et al., 2003). The P_{97} promoter contains a TATA box and a binding site for stimulatory protein-1 (Sp1), which is the principal factor activating the promoter (Apt et al., 1996). Two

binding sites for E2, with sequence ACCGNNNNCGGT, are located between the Sp1 site and the TATA box (Demeret et al., 1997). Papillomaviruses outside the alpha-group are regulated in different ways as they normally lack the Sp1 and E2 sites (Kalantari & Bernard, 2006). Late viral transcription is activated on epithelial differentiation from start sites located within the E7 ORF. For HPV-31, this promoter has been named P_{847} (Ozbun & Meyers, 1997).

1.7 Proteins of papillomaviruses

The E1 protein is a 70-80 kDa phosphoprotein with DNA-dependent adenosine triphosphatase (ATPase) and DNA helicase activity (Wilson et al., 2002). E1 binds in the URR at the the origin of replication, which consists of a direct repeat, an AT-rich region and an E2 binding site. E2 proteins enhance E1 binding and together are essential for viral DNA replication (White et al., 2003).

The E2 protein is a 40-45 kDa phosphoprotein consisting of three functional domains, a transactivator, a DNA binding domain and a hinge region (Morgan & Donaldson, 2006). The function of the hinge region is unknown but its phosphorylation regulates its turnover (Penrose et al., 2004). The transactivator region binds to 12 bp palindromic DNA sequences within the URR and shares a similar structure to the Epstein Barr Nuclear Antigen, which is the viral origin of recognition factor for EBV (Morgan & Donaldson, 2006). The binding of E2 protein to the URR represses E6 and E7 transcription by sterically interfering with the binding of transcription factor TFIID and/or human RNA polymerase II to the TATA box (Enzenauer et al., 1998). This repression works through a negative feedback loop since elevated levels of E2 are seen with E6 and E7 (due to

polycistronic E6/E7/E2 mRNA), which then act to repress transcription from URR (Morgan & Donaldson, 2006). The transcriptional regulation by E2 is further complicated by the detection of truncated forms of the protein, which retain the DNA binding domain but lack the transcription activation domain and therefore function solely as a repressor (Kalantari & Bernard, 2006).

In any case, the repression of E6 and E7 is thought to aid the switch from early to late mRNA synthesis during the viral life cycle, under permissive conditions (Severson et al., 2001). During progression of infected cells to malignancy, higher levels of E6 and E7 expression have been observed and it is thought to be due, in part, to the integration of the viral genome into the host sequence (Schneider-Maunoury et al., 1987). Integration has been observed within the E2 ORF and this can be expected to result in the loss of the repressive action of E2 on E6 and E7 expression (Park et al., 1997). However, HPV-16 DNA has remained episomal in some tumors and cell-lines. Therefore, viral DNA integration is not a necessary event for transformation (Cullen et al., 1991).

The E5 protein has been found to be weakly oncogenic (Oelze et al., 1995). It has been found to increase the duration and scope of HPV-16 infections and this is thought to be its role in malignant progression (Suprynowicz et al., 2006). The HPV-16 E5 protein contains 83 amino acids and is strongly hydrophobic (Tsai & Chen, 2003). It associates with the Golgi apparatus, endoplasmic reticulum and nuclear membrane in host cells (Conrad et al., 1993). Bovine papillomavirus E5 proteins have been found to downregulate surface expression of major histocompatibility complex (MHC) class I, which is expected

to enhance the ability of infected cells to evade detection by the host immune system (Suprynowicz et al., 2006).

E4 is a major regulator of the productive phase of the viral life cycle but its role is likely pleiotropic (Roberts, 2006). E3 and E8 have no known function and are only present in a small number of papillomaviruses (Sanclemente & Gill, 2002).

The major capsid protein of papillomavirus, L1, assembles into virus-like particles (VLPs) and these have been the subject of prophylatic vaccine development since the early 1990s (Rose et al., 1993). VLPs have certainly aided the development of enzyme-linked immunosorbant assays (ELISAs) and virion structure since no X-ray crystallographic structure has been determined for an intact virion (Chen et al., 2001). The papillomavirus capsid surrounds a nucleohistone core and is composed of major and minor capsid proteins, designated L1 and L2 respectively (Roden & Viscidi, 2006). The capsid has a T=7 icosahedral symmetry and is composed of 72 pentameric capsomers (Baker et al., 1991).

EBV codes for several proteins that have proliferative effects on cells and the sequence of events that lead to the malignant state is multifaceted and largely unknown (Longnecker, 1998). Whereas for HPV, with oncoproteins E6 and E7, the relative simplicity is deceptive since their pathogenesis is complex and involves the activities of viral proteins on multiple host regulatory pathways. The HPV E6 and E7 proteins modulate cellular proteins that regulate the cell cycle. Expression of high-risk HPV-16 E6 and E7 genes in human epidermal

cells effectively facilitates their immortalization (Hawley-Nelson et al., 1989). These immortalized cells display the histomorphological characteristics of high-grade squamous intraepithelial lesions, which are known precursors of cervical cancer (McCance et al., 1988). Furthermore, HPV E6 and E7 proteins dramatically augment genomic instability (Duensing & Munger, 2002). For example, specific gains of chromosome 3q have been observed at the transition from high-risk HPV associated severe dysplasia to invasive carcinoma (Habermann et al., 2004).

1.7.1 Oncoprotein E6

HPV-16 E6 is an 18 kDa protein of 151 amino acids containing two zinc fingers, which are essential for most properties of the protein (Longworth & Laimins, 2004b). E6 protein can be found in both the nuclear and cytoplasmic compartments of infected cells (Androphy et al., 1987) and HPV E6 has been shown to interact with at least fifteen different proteins (Longworth & Laimins, 2004b; Narisawa-Saito & Kiyono, 2007). The consequence of its interaction with some cellular proteins, especially p53, indicates that E6 acts in the G_1 phase of the cell cycle and facilitates cells to move into the S phase. Although the functions of E6 protein are essential for certain HPV properties (e.g. activation of telomerase in differentiated cells), not all papillomaviruses code for this protein (Pennie et al., 1993).

The cellular protein p53 is a sequence-specific transcriptional regulator, containing an N-terminal activation domain and a central DNA binding domain. Its ability to stimulate transcription of p53 responsive genes is tightly regulated. It

plays a pivotal role in cell proliferation and apoptosis (Werness et al., 1990). The importance of p53 in the response to damage or stress, such as viral infection, is emphasized by the fact that it is the most frequently mutated gene in human tumors. p53 regulates both the G_1/S and G_2/M checkpoints of the cell cycle (Shu et al., 2007). The amount of p53 is normally low but can be increased when cellular DNA is damaged (Selter & Montenarh, 1994). In response to DNA damage, p53 can activate one of two responses, leading to either G_1/S arrest or programmed cell death (apoptosis). p53 can act as a tumour suppressor since it indirectly regulates the cell cycle through p21, a cyclin kinase inhibitor (Ko & Prives, 1996). The transcriptional activation function of p53 is one mechanism by which it induces apoptosis through p53-responsive genes such as *Bax*. *Bax* facilitates apoptosis through mitochondria-mediated cell death events (Wu & Deng, 2002). The apoptotic pathway is a defense mechanism to prevent the spread of virus to neighbouring cells. Another recently identified p53 responsive gene is called *Notch1*. This gene is a particularly interesting tumor suppressor with regard to HPV induced carcinogensis because it has been shown to act as a determinate of epithelial cell differentiation and disappears in the late stages of cervical cancer (Narisawa-Saito & Kiyono, 2007). Cellular differentiation is required for a productive HPV infection.

Therefore, with viral infection and potential viral genome integration and resulting host DNA damage, p53 becomes activated and induces high-level p21 expression, which results in cell cycle arrest. However, the proposed role of E6 is to mitigate this action of p53 and thereby move infected cells into the S phase

and permit cellular differentiation. In this way, the infected cells will make infectious HPV virons as part of a productive infection. The E6 protein of high-risk HPV types binds to p53 in a ternary complex with a ubiquitin ligase called E6AP (Huibregtse et al., 1991). This complex results in the ubiquitination of p53 and subsequent degradation by 26S proteasome, which can reduce the half-life of p53 from several hours to less than 20 min (Hubbert et al., 1992). Consequently, p53 is cleared from the infected or transformed cell. In this way, E6 triggers an increased turnover of p53 and thereby inhibits p53 dependent transcription and the induction of apoptosis.

1.7.2 Oncoprotein E7

HPV-16 E7 protein is found predominately in the nucleus and contains 98 amino acids and has a molecular weight of 11 kDa. It appears that E7 binds to a variety of cellular proteins at different and important stages in the cell cycle, such as cyclins A and E and cdk inhibitors p21 and p27, and therefore can alter a number of different cell pathways (McCance, 2006). E7 proteins can also associate with histone deactylases, which can directly inactivate E2F (Longworth & Laimins, 2004a). A well documented interaction of E7 involves Rb, which is an important regulator of cell division (Scheffner & Whitaker, 2003).

Rb is either hyperphosphorylated or hypophosphorylated, and its state is coupled with the phase of the cell cycle (Weinberg, 1995). It becomes phosphorylated at multiple serine residues by cdks at the G_1/S boundary and remains phosphorylated during S, G_2 and until late M when it is dephosphorylated (Munger & Howley, 2002). Hyperphosphorylated Rb,

occurring at the end of the G_1 phase, releases E2F, which is bound to the hypophosphorylated form of Rb (Farnham et al., 1993). The binding of E7 protein to hypophosphorylated Rb has the same effect as phosphorylating Rb, i.e., release of bound E2F (Imai et al., 1991). E2F is a transactivator of many genes including DNA polymerase and therefore overexpression of E7 activates E2F-regulated genes resulting in an uncontrolled proliferation of cells (Severson et al., 2001).

1.8 Cervical cancer management

HPV warts can be embarrassing and a nuisance, but the association of HPV infection with cancer is of the greatest medical concern. HPV can potentially cause cancer of any site that it infects, but cervical cancer is the most common because the site of infection, the 'zone of transformation', is especially vulnerable. The normal cervix is covered by a non-keratinizing, stratified squamous epithelium and is at the junction between the squamous epithelium lining of the vagina and the mucus secreting columnar epithelium lining the endocervical canal.

The squamocolumnar junction can coincide with the external orifice of the cervix but often is carried out on to the anatomical ectocervix, which can expose the tissues previously found in the lower endocervical canal to the vagina (Marchionni et al., 1981). This physiological process and the exposed tissue form the cervical 'ectopy'. It is from the epithelium that covers the ectopy that most cervical intraepithelial neoplasia develop (Arends et al., 1998). The columnar epithelium of the ectopy undergoes metaplasia to a stratified squamous

epithelium, forming the so-called 'zone of transformation'. It is during this metaplastic process that the epithelium seems to be particularly vulnerable to oncogenic viruses and perhaps other factors resulting in the development of an intraepithelial neoplasm, which may be of the squamous or columnar cell type (Cox, 1995).

There are several terms used to describe cervical carcinoma and its premalignant forms. Carcinoma in situ (CIS) refers to a malignant lesion confined to the epithelium that has not invaded the underlying stroma, as opposed to invasive cervical cancer (ICC). Cervical intraepithelial neoplasia (CIN) is defined as the spectrum of intraepithelial changes from minimal cytological atypia to invasive squamous cell carcinoma. CIN is divided into three grades; CIN1, CIN2 and CIN3 (Crum, 2003). CIN1 has lesions with well differentiated neoplasm whereas CIN3 is poorly differentiated and CIN2 is intermediate between the two.

The natural history of CIN indicates that the approximate likelihood of CIN1 regression is 60%, persistence 30%, progression to CIN3 10% and progression to cancer 1% (Iatrakis et al., 2004; Ostor, 1993). HPV-induced carcinoma can develop within 2 years after initial infection but most cancers develop from precursor lesions that progress from one stage to the next over 10 to 30 years (Ghim et al., 2002). It has been shown that 99.7% of all cervical carcinomas are positive for HPV DNA establishing that HPV infection and its sequela are necessary for almost all ICC (Walboomers et al., 1999).

1.8.1 Prevention

Primary approaches to prevent HPV infection include risk reduction and vaccination. Immunization against HPV infection is essentially cancer prevention and should not be confused with cancer vaccination or the specific action of immunotherapy of cancer. Prophylactic vaccines generate antibodies that neutralize the viral inoculum prior to infection whereas therapeutic vaccines would treat established infections by eliciting an immune response against infected cells, thereby eliminating lesions or malignancies. Consequently, an HPV therapeutic vaccine would work against the antigens that would be expressed during infection and not, necessarily, those produced by cervical cancer cells. The recently approved HPV vaccine is prophylactic and involves immunizing females before they become sexually active with the goal of eradicating the incidence of disease.

HPV infection does not cause a systemic infection or kill the infected epidermal cell and often induces no inflammatory response. Only 50% of those becoming infected with HPV (i.e., HPV DNA positive) show a neutralizing antibody response while the other half clear the infection without a measurable antibody response (Carter et al., 2000). Knowledge about the natural clearance of HPV infection suggested that neutralizing antibodies may not be sufficient for protection but rather required a strong cell-mediated response (Coleman et al., 1994). However, a trial of an HPV-16 prophylactic vaccine demonstrated a 99.7% seroconversion rate and 100% efficacy for the prevention of cervical intraepithelial neoplasia (Koutsky et al., 2002). Consequently, it appears that

cell-mediated immunity is important in clearing an infection, but humoral immunity is sufficient in protecting an individual from becoming infected (Giles & Garland, 2006).

One recently developed prophylactic vaccine is tetravalent containing L1 VLPs (Roden & Viscidi, 2006). Prophylatic vaccines typically use live attenuated or inactivated viruses which was not possible with HPV since the virus cannot be efficiently propagated in cultured cells (Hagensee & Galloway, 1993). However, the use of VLPs circumvented the problem since they can be overexpressed in yeast and induce titers of serum neutralizing antibodies equivalent to those generated with native virion (Roden & Viscidi, 2006).

Clincial trials with the prophylactic HPV vaccine called Gardasil® (Merck Frosst) demonstrated that it successfully prevented cervical cancer precursors, cervical intraepithelial neoplasia, and anogenital warts caused by HPV types 6, 11, 16, and 18 (Siddiqui & Perry, 2006; Villa, 2006). It was licensed by the US Food and Drug Administration (FDA) in June 2006 and approved by Health Canada on July 10, 2006 for use in females aged 9-26 years (Dunne & Markowitz, 2006). The Advisory Committee on Immunization Practices (ACIP) recommended the vaccine for three age groups: all girls between 11 and 12; and women 13 to 26 who have not received the vaccine yet; and women who have had abnormal Pap smears or genital warts.

1.8.2 Screening

The mantra within the medical community is to find cancer at an earlier stage by identifying the initial molecular and cellular changes that occur in

malignant cells. The idea is that these genetic and antigenic changes will serve as biomarkers for early cancer detection and for risk assessment. Unfortunately, many of the clinical tests currently in use are not sufficiently sensitive or specific to detect cancer or to evaluate risk accurately enough to guide effective preventive interventions. Similarly, effective screening for cervical cancer goes beyond the laboratory test to the establishment of a comprehensive and organized program that is intended to identify groups of asymptomatic women with precursors of cervical cancer. A typical program involves education, recruitment, screening for precursors and triage of women for a definitive diagnosis so that these women can be queued for treatment. As of September 2006, Saskatchewan was the only province in Canada with a fully implemented program, which has recruitment, recall, follow-up and data collection systems in place (Murphy, 2007).

The Pap smear cervical cytology screening test is the basis for most cervical cancer screening programs and is one of the most effective cancer prevention strategies. Cervical screening programs using Pap tests are being credited for reducing the incidence of cervical cancer (Liu et al., 2001). It has been estimated that without the Pap test the number of cervical cancer cases would be 75% higher (Robles et al., 1996). However, an audit of the UK program found that 47% of women under the age of 70 with stage IB cervical cancer or worse occurred in individuals with an adequate screening history (Sasieni et al., 1996). In any case, most cervical cancer deaths are seen with women who have never had a Pap test. In Canada, this demographic is estimated to be over 10%

of women aged 20 to 69 years (Canadian Community Health Survey 2003). These women tend to be those that didn't have a regular physician, were 20-29 years of age, had a lower income, had less education and were a member of a visible minority (Murphy, 2007).

The current definitive diagnostic test for cervical cancer is the colposcopically directed biopsy (Kendrick et al., 2007) and is the diagnostic test used in Saskatchewan (Dr. L.A. Brydon, personal communication). Histology of these biopsies will reveal the architectural arrangement of abnormal cells whereas the clinical examination will ensure adequate sampling of the lesion. The application of acetic acid to the cervical epithelium is often useful in revealing lesions since genital HPV infections are often flat and invisible to the unaided eye (Paavonen et al., 1988; Singh et al., 2001). Consequently, women with abnormal Pap smears are often followed rigorously and repeat abnormal Pap smears or highly abnormal Pap smears typically trigger the triage of these women to colposcopy for histological confirmation of disease.

Colposcopically directed biopsy has its limitations. Meta-analysis studies of 86 articles between 1960 and 2000 has shown that colposcopically directed biopsy for the detection of any grade of CIN has a sensitivity of 96% and a specificity of 48% (Mitchell et al., 1998). It has been concluded that multiple and random placement of biopsies are better than the colposcopically directed biopsy since small-volume lesions of CIN2 and CIN3 are common and likely missed by colposcopy (Jeronimo & Schiffman, 2006).

More recently, HPV detection technology, specifically HPV DNA testing, has been studied to evaluate its potential role in three main areas of cervical cancer testing: 1) triage of cases with minor cytological abnormalities or abnormalities of undetermined significance, 2) follow-up after treatment of CIN and, 3) for primary screening as an alternative or adjunct to Pap tests.

The utility of HPV DNA testing for triage of atypical squamous cells of unspecified significance (ASCUS) has been well studied with perhaps the most thorough data set coming from the ASCUS/LSIL Triage Study (ALTS). ALTS provided longitudinal data by following women with an original report of ASCUS every 6 months over a period of two years and followed up with colposcopy (Schiffman & Adrianza, 2000). The results from ALTS have lead to recommendations that HPV DNA testing is useful for managing women aged 30 or older with ASCUS (Provencher & Murphy, 2007). On the other hand, 83% of women with low-grade squamous intraepithelial lesion (LSIL) in the ALTS trial were HPV positive and this low specificity lead to recommendations that HPV DNA is not useful for triage of these women (Arbyn et al., 2006).

Women treated for CIN must have follow-up to monitor outcome. Recurrent CIN averages 10% after 2 years and is more common in older women (Arbyn et al., 2006). HPV testing has been shown to pick up residual disease quicker and with higher sensitivity and similar specificity compared to follow-up with Pap tests (Arbyn et al., 2006; Bae et al., 2007; Nanda et al., 2000).

Detection of HPV DNA is more commonly associated with CIN2 or CIN3 than Pap testing (95% versus 84%) but is less specific (60% versus 85%) and

this has lead to its rigorous evaluation as a screening test (Arbyn et al., 2006). However, the reduced specificity of HPV DNA testing is expected to increase the number of women needing follow-up with either Pap testing or colposcopy (Clifford et al., 2006). A recent study suggests that for women 30 to 69 years the specificity of HPV DNA was 94.1% while it was 96.8% for Pap testing (Mayrand et al., 2007). In combination with Pap testing as a primary screen for cervical cancer, testing for HPV DNA can help reduce the risk for CIN2 or CIN3 by approximately 40% for women in their mid-30s (Naucler et al., 2007). The benefit of the increased sensitivity of HPV DNA testing is the enhanced negative predictive value, which has the potential to allow a safe increase in the screening intervals for HPV DNA negative women. This advantage may be more of a requirement for screening programs with the advent of the HPV vaccine as a prevention strategy. The HPV vaccine may impact Pap screening effectiveness with women either foregoing the test or due to the pressure of potentially competitive prevention and screening programs on the same public health budgets (Franco et al., 2006).

1.8.3 HPV detection technologies

The potential use of virus-specific antisera is limited because viral protein production is dependent on whether the lesion is productive or malignant (Doorbar, 2005). Furthermore, assays using virus-specific antisera cannot differentiate between current and past infection (Nonnenmacher et al., 2003). Overall, HPV antibodies in women are not well correlated with HPV infection or the development of CIN (Nonnenmacher et al., 2003; Vonka et al., 1999). Anti-

HPV humoral immune responses are generally measured by enzyme-linked immunoabsorbent assay (ELISA) with HPV type-specific virus-like particles (VLPs) absorbed in plates (Iftner & Villa, 2003). ELISAs show sensitivities of approximately 60% with specificities of 90% (Strickler et al., 1998). However, recent advances in multiplex serology using Luminex technology shows promise but will be limited to population-based research studies (Waterboer et al., 2005).

Individual viruses cannot be detected by light microscopy but their cytopathic effects are often visible. The Pap smear is the most common technique for the diagnosis of cervical cancer and its precursors. Sampling of the cervical transformation zone is an integral part of the Pap smear. The Pap smear is a preparation, on a glass slide, of cervical cells obtained by abrasion (e.g., brush), fixed, by either spraying or immersing the slide into 95% ethanol, methanol or isopropanol, and then stained with the Pap stain. On a Pap smear, HPV-infected cells are termed koilocytes, from Greek 'koilos' meaning hollow/cavity. Koilocytes have a marked density of cytoplasm peripheral to a cavity, amphophilic cytoplasm and an enlarged hyperchromatic nucleus (Bollmann et al., 2005).

A systematic review to determine the accuracy of the conventional Pap test found that most studies are severely biased but the best estimates suggest that it has a sensitivity that ranges from 30% to 87% and a specificity from 86% to 100% (Nanda et al., 2000). Despite the advantages of the Pap test it has been concluded that it will remain an imperfect diagnostic science due mainly to sampling error and to interpretive subjectivity of the cytopathologist

(Chantziantoniou, 2007). Advances made using liquid-based cytology, where cells are collected in liquid rather than on a glass slide as with conventional Pap testing, has reduced the number of false negative results but not for high-risk populations (Runowicz, 2007).

HPV DNA detection has an advantage over Pap testing since it will also detect normal appearing cells next to the lesion that contain latent papillomavirus (Ferenczy et al., 1985). For this reason and others, it is substantially more sensitive than the Pap test in detecting high-grade CIN but significantly less specific. HPV DNA testing using a commercial assay called Hybrid Capture™ by Digene Inc. has been approved by the US Food and Drug Administration for use as an adjunct to cytology and has been recommended for cervical cancer screening of women aged 30 years or more (Wright et al., 2004). For women over 30 years, the sensitivity of Hybrid Capture was 94.8% and the specificity 86.0% for the detection of high-grade CIN (Koliopoulos et al., 2007). The addition of amplification of HPV DNA by the polymerase chain reaction (PCR) results in a substantial increase in test sensitivity. A commercial PCR-based assay using a nondegenerate pool of primers for the L1 gene against 13 high-risk genotypes has been developed by Roche Diagnostics and shows good agreement compared to Hybrid Capture (Carozzi et al., 2007). However, both these commercial assays are designed for a restricted set of HPV types and cannot provide information on the actual HPV type detected and are limited in their ability to detect multiple infections (Iftner & Villa, 2003).

A typical generic PCR assay for HPV can reproducibly detect 10 fg of HPV DNA whereas Hybrid capture requires 75 fg (Iftner & Villa, 2003). A number of PCRs for HPV DNA testing have been described and their utility for cervical cancer screening is now well established (Walker et al., 2006; Zuna et al., 2005). One approach is to first amplify HPV DNA using consensus primers designed for a broad spectrum of HPV types by targeting the highly conserved L1 ORF (Snijders et al., 2005). False-negatives due to integration events affecting the HPV L1 gene have been demonstrated (Karlsen et al., 1996; Walboomers et al., 1999). For this reason, HPV DNA negatives by L1 PCR can be queued for a second target such the E1 gene (de Roda Husman et al., 1995; Ylitalo et al., 1995). Amplicon detection and HPV type determination is then performed using agarose gel electrophoresis and DNA sequencing respectively.

Not all PCR-based methods perform equally well in the detection of multiple infections. For example, it has been shown that the GP5+/GP6+ primer set detects 47% of mixed HPV infections while MY09/11 primer set can detect 90% (Qu et al., 1997). Up to 40% of HPV infections are mixed infections and so the accurate identification of high-risk HPV genotypes in mixed infections is important for defining a woman's risk for progression to cervical cancer (Oh et al., 2007). Luminex-based HPV genotyping, which combines polymerase chain reaction amplification with hybridization to fluorescence-labeled polystyrene bead microarrays is showing excellent potential for simultaneously detecting the presence of multiple HPV types (Oh et al., 2007; Schmitt et al., 2006b). Similarly, oligonucleotide microarray-based detection systems are showing

potential for mixed infections but are currently prohibitively expensive for clinical use (Gheit et al., 2006).

The detection of HPV mRNA instead of DNA is an alternative diagnostic approach for detection of oncogene activity related to the development of CIN. A commercial assay based on this approach is called PreTect HPV-Proofer. It is a multiplex nucleic acid sequence based amplification (NASBA) assay that utilizes molecular beacon probes for real-time detection was developed for the identification of E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45 (Molden et al., 2007). It is limited in the number of HPV types detected but may serve as a valuable tool in monitoring HPV infections that produce proteins with a transforming potential.

1.8.4 Treatment

Treated HPV infections experience substantial recurrence rates (Gall, 2001). Furthermore, most HPV-induced cervical cell changes are transient and 90% regress spontaneously within 36 months (Chua & Hjerpe, 1996). Consequently, the decision to treat is dependent upon the grade of neoplasia that is found.

Interventions are composed of both tissue ablation and cytodestructive modalities. Physically ablative therapies include cyrotherapy, laser therapy, electrosurgery and surgical excision. Loop electrosurgical excision procedures (LEEP) are now considered the preferred treatment for noninvasive squamous lesions (Burd, 2003). LEEP uses an electrically charged wire to excise the transformation zone and preserves the tissue for histogical examination.

Microinvasive cancers are managed by excisional cone biopsy whereas invasive cancers require radical hysterectomy or external-beam high-energy radiotherapy and implants loaded with ^{192}Ir. Cytotoxic agents that treat genital warts destroy the affected area either by chemodestructive or antiproliferative modes of action. Cytotoxic agents include podophyllin, pdofilox, trichloroacetic acid and 5-fluorouracil.

1.9 Rationale for the research

In women, infection with HPV, considering all possible types, achieves a lifetime cumulative incidence of up to 70%, whereas cervical cancer is a relatively rare disease, with a lifetime incidence range of 1.1-3.0% across the world (Koutsky, 1997; Parkin et al., 2005). Consequently, HPV infection is extremely common but progression to serious disease is extremely rare. Moreover, the majority of women with genital HPV never develop cervical precursors.

Nevertheless, the consequence of missed diagnoses (i.e., false-negative diagnostic test results) will likely contribute to 200 cervical cancer-related deaths in Canada during 2006. In 2002, cervical cancer caused 273,505 deaths worldwide, and made it the second most common cancer among women (Parkin et al., 2005). On the other hand, false-positives in screening lead to unnecessary colposcopies, biopsies, ablational treatments, which increase both healthcare cost and morbidity. Healthcare costs are significant; more than $2 billion per year is spent in the United States on the treatment of cervical cancer (Brown et al., 2001). Certainly, monetary costs are only part of the equation. False-positive results also cause undue stress on the patient's mental health. Despite

the problems, cervical cancer caused by HPV is considered preventable in developed countries and has one of the best prognoses of all cancers with its highly successful treatment options (Parkin et al., 2005; Rydstrom & Tornberg, 2006). Certainly, cervical cancer can be detected and treated successfully but improvements in early diagnosis are required.

It is estimated that current technology could prevent up to one-third of new cancers and increase survival for another one-third of cancers detected at an early stage (Ngoma, 2006). To achieve this, the World Health Organization (WHO) has developed a comprehensive approach to cancer control, which includes a priority on cervical cancer screening and treatment (Ngoma, 2006). A similar approach has recently been taken with the Saskatchewan Cancer Agency's Prevention Program for Cervical Cancer, which is largely about educating and reminding women to have their Pap test done (http://www.scf.sk.ca).

However, a cervical cancer screening program based on Pap testing has its limitations. A Pap test is very specific for determining the presence of neoplastic transformation but it is not very sensitive (Clavel et al., 2001). In gynecologic oncology, a review of pathological reports found reproducibility to be only 84% with 2% of the corrected diagnoses having a consequential impact on proper treatment decisions for the individual patient (Santoso et al., 1998). There is significant reporting variation with diagnosis of CIN and differentiation between normal tissue and borderline abnormalities (Creagh et al., 1995). In Canada,

colposcopic examinations are highly subjective and result in non-evidence based patient management (Nelson et al., 2006).

The sensitivity of HPV DNA testing in detecting CIN2, CIN3 or invasive cancer is significantly better than cervical cytology. A meta-analysis comparing the performance of the two tests in seven different countries has found that HPV DNA testing can be up to 41% more sensitive for the detection of high-grade squamous intraepithelial lesions (HSIL) (Ghim et al., 2002). Although 99.7% of cervical cancers possess HPV DNA, the simple detection HPV DNA is a poor predictor for the risk of cancerous transformation (Clavel et al., 2001; Walboomers et al., 1999). There is strong correlation between certain high-risk HPV types and cervical cancer (Munoz et al., 2003). However, HPV-infection with a high-risk type does not present as a reasonably useful marker for transformation since the majority of infections are transient and persistent infection is required for transformation (Cuschieri et al., 2005). To date, no biomarker has been found which reveals the 'persistence' of HPV infections.

It is known with *in vitro* studies that transformation by HPV requires expression of E6 and E7 ORFs (Ueno et al., 2006; Watanabe et al., 1989). Normally, transcription of the genes for capsid proteins is restricted to terminally differentiated epidermal cells (Stoler et al., 1989). It has been observed that as the grade of neoplasia is increased, cellular differentiation is decreased and L2 and L1 transcripts become undetectable (Stoler et al., 1992). HPV-induced immortalization can be accompanied with integration of the viral DNA into the host cell genome. An integration event that causes disruption of the E2 gene is

followed by overexpression of the E6 and E7 oncoproteins (Schmidt et al., 2005). Consequently, integration and/or malignant transformation is expected to be accompanied by high levels of E6 and E7 transcripts and a reduction of L1 transcript and viral DNA.

1.9.1 Research objectives

Although the biochemical and molecular basis of HPV's role in carcinogensis has been extensively studied *in vitro*, I set out to extend these findings *in vivo* and determine the utilty of HPV typing and relative quantification of HPV-16 transcripts for monitoring HPV vaccine efficacy and improving colposcopy triage of women with abnormal Pap smears. The research work had two main objectives that are focused on aspects of HPV and its role in cervical disease. First, to determine the distribution of HPV types and the epidemiology of HPV infection in a population of Saskatchewan women referred to a colposcopy clinic (discussed in 3.1). Second, to examine the potential of HPV-16 transcripts and HPV viral load for the detection of CIN (discussed in 3.2). These objectives were bracketed with extensive laboratory work and statistical analyses for both method development and validation (discussed in 3.3).

The first objective of the research was to determine the distribution of HPV types and to describe the epidemiology of HPV infection in Saskatchewan. Most studies have found that the vast majority of cervical cancer cases are caused by persistent infection with the high-risk types 16 and 18. Pooled analysis of 3,085 cases of cervical cancer from 25 countries found that HPV-16 was found in 57% of cases with 17% having HPV-18 (Clifford et al., 2005a). On the other hand,

most genital warts, in general, are caused by HPV types 6 and 11 (Clifford et al., 2005a). For this reason, vaccine development has focused on these four HPV types (Siddiqui & Perry, 2006).

Clinical trials with the HPV vaccine called Gardasil® (Merck Frosst) have had success in preventing cervical cancer precursors, cervical cancer, and anogenital warts caused by these HPV types (Siddiqui & Perry, 2006; Villa, 2006). The approved HPV vaccine is licensed by the FDA for use in females aged 9-26 years (Dunne & Markowitz, 2006). A second vaccine, covering only types 16 and 18, is being developed by Glaxo Smith Klein and it has been shown equally effective in clinical trials (Harper et al., 2004; Harper et al., 2006). However, heterogeneity in HPV type distribution should be taken into account when predicting the effect of vaccines on the incidence of infection or in developing screening tests for the virus. Replacement disease caused by serotypes of *Streptococcus pneumoniae* not covered by the polyvalent conjugate vaccine was shown to be a negative factor in the prevention of invasive pneumococcal disease (Daily, 2005). Similarly, it is a reasonable concern that as the HPV vaccine becomes widely used, other high-risk HPV types may replace any high-risk HPV types covered by the vaccine. Consequently, newer polyvalent vaccines may need to be developed. The choice for which high-risk HPV types should be included needs to be balanced with prevalence rates and risks posed for CIN2 or worse.

The frequency of HPV-31 detection in Saskatchewan was noted to be higher than in other Canadian provinces or worldwide (Dr. A. Severini, personal

communication). One intention of this research was to determine if the prevalence pattern of HPV-31 was substantial and sustained in the study population. A high prevalence of HPV-31 could have implications for utilization of future vaccines and management of cervical disease. In any case, effective public health requires the knowledge of HPV genotypes and specifically the distribution of high-risk types.

The second objective of this research focused on viral transcript levels and its prospective role in carcinogenesis. An increase in the relative amounts of E6 RNA, E7 RNA or a decrease in L1 RNA in cervical samples may correlate with the risk of cancerous transformation. The specific examiniation of L1 RNA is another unique aspect of the research. Previous work has demonstrated that the amount of HPV E6 and E7 mRNA is associated with the presence of CIN (Czegledy et al., 1994; Daniel et al., 1995; Hsu et al., 1993; Ke et al., 1999; Park et al., 1997; Selinka et al., 1998; Sotlar et al., 1998; Sotlar et al., 2004; Wang-Johanning et al., 2002). However, these studies reveal that the predictive value of HPV RNA remains low.

The lack of predictive power could be attributed to one or more problems with the research to date: low sample number, inappropriate use of technology, poor quality assurance and insufficient control for variables, or to intrinsic variation of E6 and E7 RNA levels in cervical samples. This study extends previous studies by taking a different approach while also utilizing rigorous quality control to ensure the best relative quantification as technologically and practically possible. This has not been done before. Details on how

papillomaviruses directly activate the transcription of genes or alter the activity of cellular factors are poorly understood. Furthermore, many studies investigating the function of HPV gene products have not been done within the context of so many viral transcripts, HPV viral load and with relation to host nucleic acids.

This study was expected to resolve the question as to whether measurement of viral transcripts has diagnostic value for cervical cancer. It used the unique approach to relative quantification (RQ), in which the levels of HPV transcripts and DNA are standardized by the levels of host DNA (β-actin), host RNA (S9 transcripts) or viral load.

The appropriate use of real-time RT-PCR is providing unprecedented utility in the quantification of mRNA (Bustin, 2002; Bustin & Mueller, 2005; Stahlberg et al., 2005). Using real-time RT-PCR technology, this study sought to determine the relative amounts of E6, E7 and L1 transcripts from HPV-16 positive cervical scrapings. Furthermore, measurement in relation to total human nucleic acid and viral DNA were expected to correct for the variables such as proportion of HPV-infected cells present in cervical samples and RNA degradation. Assay precision was enhanced by performing determinations in triplicate. Kinetic outlier detection was implemented. Sample numbers were more than adequate and power of resolution assured. Data were rigorously examined with the use of appropriate statistics.

It would be of notable clinical significance to find an HPV marker for cervical cancer. Such a marker could serve as the basis for an adjunct test to Pap smear screening for colposcopic triage. Ideally, finding a single test which

has the all the required diagnostic performance characteristics would be preferred. However, an improvement in colposcopy triage practice may be achieved by using different tests in combination, particularly when no single test is satisfactory for both specificity and sensitivity. Furthermore, judicious use of available testing for triage could eliminate unnecessary colposcopy or treatment. A well-designed cascade of molecular tests as a follow-up to Pap screening of the general population could identify women that need further follow-up at colposcopy, while increasing the screening period for women who are not at risk.

In other words, a direct benefit of the thesis work could be to lay the foundation for the development of a molecular test capable of detecting the initial stages of neoplastic transformation by HPV. In essence, this focus is aimed at establishing a marker for cervical cancer. This study also sought to correlate measurement of viral transcripts with the cervix histology to identify a pattern, which is common to or very strongly associated with CIN and cancer. It was expected that a number of samples from normal cervices would share the cancer-associated amount of transcript, either because they have still not developed CIN, or because the Pap smear examination failed to detect it. Patients in this study could be candidates for a future prospective study to determine if high levels of HPV RNA indicate a risk of developing CIN and cancer. Such a study could be used to examine the prognostic value of viral transcripts.

2. MATERIALS AND METHODS

2.1 Study subjects

The Saskatchewan Cervical Cancer Prevention Program encourages women, ages 18 to 69 years, to have regular Papanicolaou (Pap) smear testing every three years, or for women who have never had a Pap test to have two Pap tests, one year apart. Patients with abnormal or indeterminate results on their routine Pap smear test are then typically referred for clinical management by a gynecologist. Gynecologists may opt to examine women with abnormal Pap results in a colposcopy clinic, which facilitates the visual examination of the cervix, biopsy collection and treatment as indicated. Specimens for this study were obtained from women who were referred to the colposcopy clinic at the Women's Health Centre located at the Regina General Hospital.

The study subjects were categorized based on the time of specimen collection for HPV testing. Specimens collected from January 1998 through September 2003 were identified as retrospective study subjects, whereas prospective study subjects were those who had their specimens collected from October 2003 through February 2005. The retrospective study subjects utilized specimens that were archived at the National Microbiology Laboratory. The specimens were originally collected for HPV testing and were genotyped using Southern hybridization technology. The archived samples were retested using the same DNA sequencing protocol as was used for the specimens from the prospective study subjects. The HPV typing was repeated for the archived specimens because it was expected that the probe specificity used with the

Southern hybridization technology was questionable. No provisions were made to preserve the RNA in the archived specimens and consequently no measure of transcript levels were made with the retrospective study subjects.

There was a significant difference between the specimen acquisition criteria from the retrospective and prospective study subjects. Samples from the retrospective study subjects were collected as part of the regular medical workup and DNA extracted from each specimen was archived at -70°C. Consequently, cervical scrapings from the retrospective study subjects were only submitted for HPV testing as deemed medically appropriate by the physician. On the contrary, the prospective study period captured >99% (785/790) of the eligible women who were referred to the colposcopy clinic. Patients were only eligible to participate if they were under the guidance of a gynecologist that was enrolled as a collaborator in the study. Initially, one gynecologist was enrolled and patients under her care were recruited starting October 10, 2003. Once the collection protocol was established within the clinic, seven other gynecologists from the Regina Qu'Appelle Health Region were enrolled on February 2, 2004. The recruitment phase was discontinued on February 22, 2005 when 785 study specimens were collected for the prospective portion of the study.

Informed consent for the collection of prospective study specimens from the patient was obtained by the enrolled physician using consent forms (Appendix A), which explained the purpose of the study, the voluntary nature of participation, assurance of confidentially, risks and benefits of enrollment, and contact information for the investigators if more information was desired.

Informed consent was indicated with a signature on a perforated portion of the consent form that was witnessed and collected by the attending nurse or physician. The study subject recruitment strategy and procedures to ensure patient anonymity were approved by research ethics boards at both the University of Regina (Appendix B-1) and the Regina Qu'Appelle Health Region (Appendix B-2) and were renewed annually until the recruitment phase was complete. The director of the colposcopy clinic was the only individual who could link the de-identified study specimen to the patient.

2.2 Specimen collection

Patients at the colposcopy clinic were queued for medically appropriate examination and treatment by a gynecologist, which typically included another Pap test and visual examination of the cervix. For consenting patients, a study specimen was taken immediately after the Pap test swab. Cervical cells for the study were collected using a Cervex-Brush® (Rovers Medical Devices), which is capable of removing endocervical and ectocervical cells simultaneously. The presence of both cell types indicates that the transformation zone, where precancerous changes occur, has been correctly sampled. The deliberate pursuit of both cell types due to the brush head design was important since specimen adequacy, which is typically determined by a cytologist, could not be accessed with the study specimens.

Immediately after collection, the brush head of the Cervex-Brush® was removed from the shaft and placed into a sterile disposable 50 ml centrifuge tube containing 5 ml of cold 95% ethanol. Tubes containing brush heads were labeled

with a study number and then placed on dry ice. Study specimens were labeled sequentially with study numbers that were preprinted on self-adhering freezer-safe labels. The same study number was affixed to the medical record of the patient and to the signed consent form. The study specimens were frozen in ethanol as quickly as possible to preserve the nucleic acids for subsequent detection and relative quantitative analysis. Cervical cells were kept on dry ice for shipping to the Molecular Diagnostics section of the Saskatchewan Provincial Laboratory (DNA lab) within 3 h of collection. Hereafter, data generated from the DNA lab was termed 'research data'. At the DNA lab, each specimen was only identifiable by the collection date, attending physician and study number. This information was used to match research data on the study specimen to all other non-identifying patient information and to report HPV-genotyping results to the submitting physician.

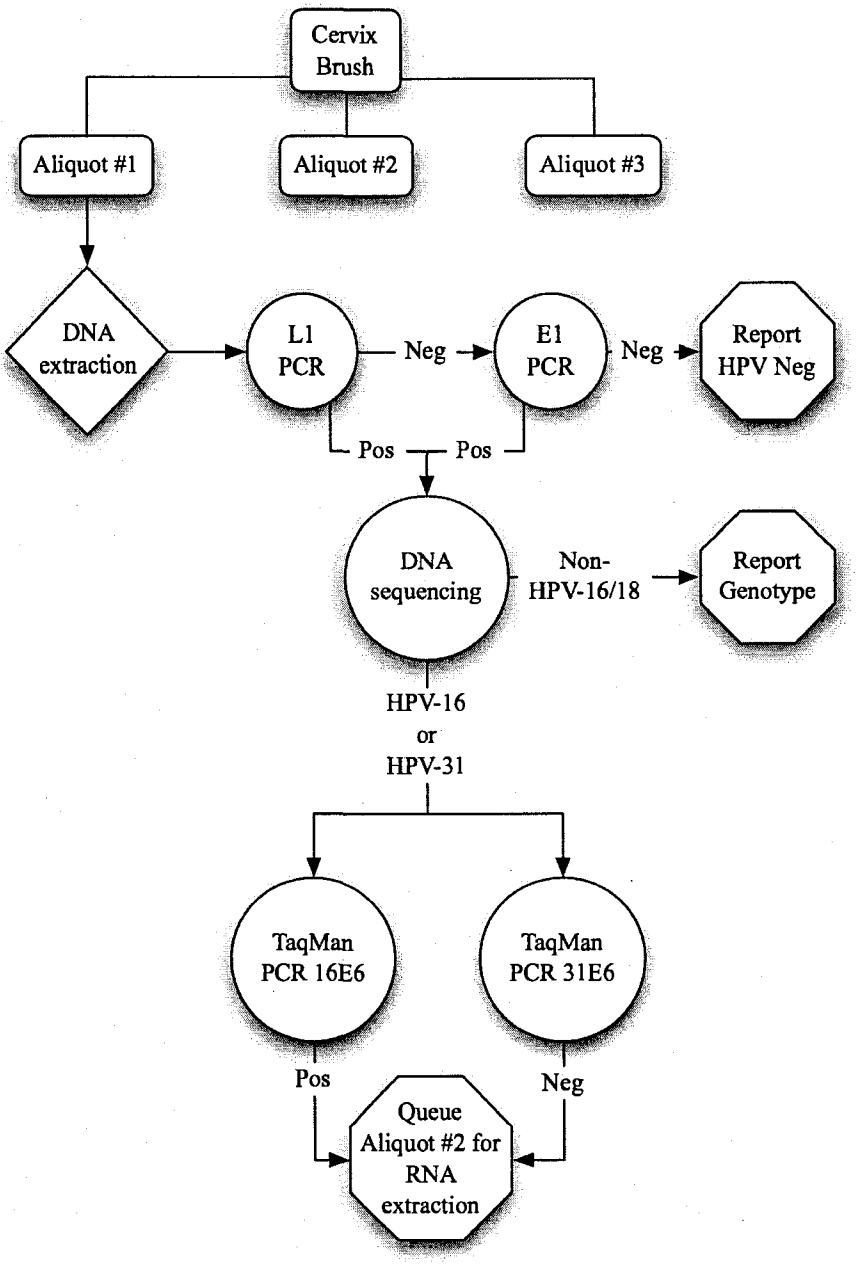


Figure 2. Specimen workflow - DNA processing schematic.

Aliquots of cell suspensions were processed to ensure nucleic acid stability while minimizing workup of either negative samples or non-HPV-16 positive specimens. This schematic depicts how HPV-16 positive specimens were screened and confirmed. The real-time PCRs were used as a final confirmation of HPV type and to identify any HPV-16 positive specimens that were co-infected with HPV-31.

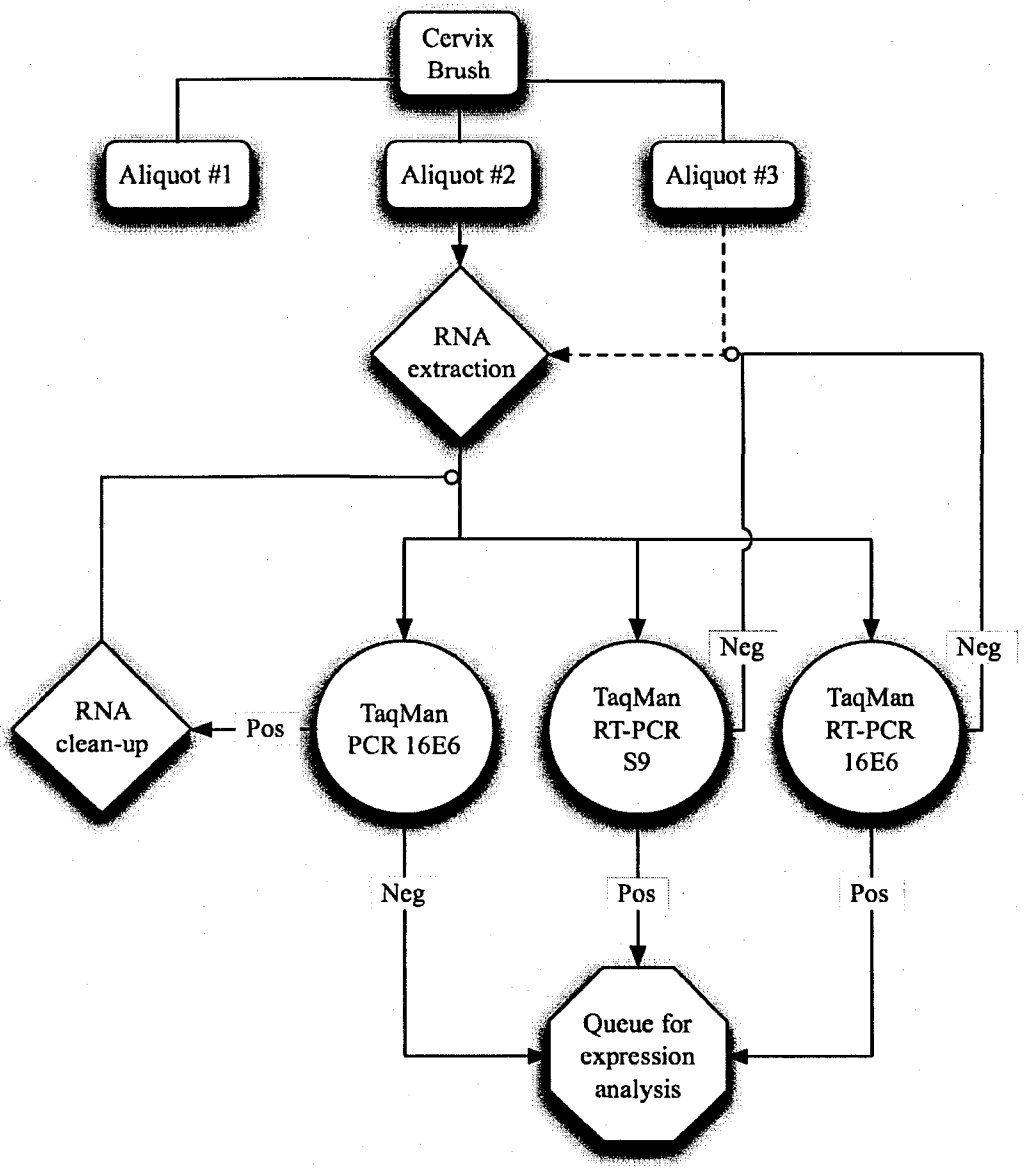


Figure 3. Specimen workflow - RNA processing schematic.

This schematic depicts how cell pellets, that were determined to contain HPV-16 only, were processed for the RNA fraction. RNA was queued for relative quantification if sufficient quantity of human and viral RNA was detected and no viral DNA contamination of the RNA fraction was found by real-time PCR. The third aliquot was only processed if the quality control parameters had failed with the second aliquot.

2.3 Specimen processing

Upon arrival at the DNA lab, all specimens were kept on dry ice until processing, which took place within 4 h. Study specimens were removed from the dry ice and immediately pulse-vortexed to remove cells from the brush head until the suspension appeared homogeneous, typically 1 min. Three, 1 ml aliquots of the cell suspension were transferred to O-ringed screw-capped microcentrifuge tubes that were pre-chilled in an aluminum block kept at -70°C until needed. The aliquots were spun at 3000 x *g* for 5 min at 4°C. The centrifuge was stopped using reduced deceleration to avoid resuspension of the pellet from braking action. The supernatant was aspirated with a pipettor and the remaining cell pellet was disrupted in the appropriate lysis buffer. The first aliquot was queued for DNA processing and analysis as indicated in Figure 2. The second and third aliquots were queued for RNA processing as indicated in Figure 3.

2.3.1 Laboratory configuration and workflow

Amplification of a nucleic acid target, such as is done with PCR, increases assay sensitivity. However, laboratories that use nucleic acid amplification technologies must take precautions to prevent false-positive results that might arise from contamination of specimens by amplification products of previously analyzed specimens. All the methods described below were done in a laboratory located at the Saskatchewan Provincial Laboratory, which has an optimal facility design for PCR and accredited procedures to ensure quality control of results.

To avoid contamination, workflow was strictly unidirectional with no movement of specimens or materials in the reverse direction. Procedures were carried out in four physically separated rooms for dedicated tasks of reagent preparation, specimen preparation, reaction set-up and post-amplification analysis. The post-amplification room had 100% exhaust air with negative pressure to all other laboratories. The reagent preparation room had 100% supply air with positive pressure to all other laboratories.

Each laboratory had dedicated equipment, supplies and lab coats. Further, the specimen preparation laboratory had a bio-safety cabinet, centrifuge, heat block, pipettors and supplies dedicated to RNA extraction work. Another area within the specimen preparation laboratory was used for DNA extractions from clinical and control material. The PCR master-mixes were made in the reagent preparation laboratory and aliquotted into PCR tubes in the reaction set-up laboratory. Nucleic acid preparations were added to the PCR reagents within the reaction set-up laboratory. The ingredients for the all PCR reactions were made immediately before use and RNA was added immediately after extraction, or freshly thawed to avoid any degradation. Relative quantification reactions were setup on cooled aluminum blocks, that accommodated 96-well reaction trays. All oligonucleotide stocks and primer/probe working stocks were kept with other amplification reagents in a -20°C frost-free freezer that was separate from specimens, or extracted nucleic acids. Processing of PCR products and DNA sequencing was done in the post-amplification laboratory.

2.4 Quality control material

Cell lines derived from Human cervical cancer were used as a source for quality control material. The CaSki cell-line is reported to contain an integrated HPV-16 genome at about 600 copies per cell (Adler et al., 1997). Nucleic acids extracted from CaSki cells were used as positive amplification controls for all the PCRs except those specific for HPV-31. The CIN-612 cell-line containing copies of HPV-31 was obtained from Dr. Laimins (Northwestern University, Chicago, Illinios) (De Geest et al., 1993), and nucleic acids extracted from the CIN-612 cells were used for all HPV-31 real-time PCRs. Nucleic acids were extracted from the cell-lines using the same procedures as used for clinical specimens. Fractions of RNA and DNA from both cell-lines were kept separate and aliquots were stored at -70°C until needed. Aliquots were made for a single use in order to reduce the number of freeze-thaw cycles and thereby increase the inter-assay reproducibility. Aliquots of CaSki DNA were sufficiently diluted to be just above the limit of detection for the genotyping PCRs. Aliquots of RNA and DNA, for use as calibration controls for relative quantification, were diluted to have reasonable cycle threshold (Ct) values and within the range measured with clinical material.

CaSki cells were obtained from the ATCC (CRL-1550) and cultured as monolayers in RPMI 1640 medium with 2 mM L-glutamine and adjusted to contain 1.5 g sodium bicarbonate /l, 4.5 g glucose /l, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum at a temperature of 37°C. The subculturing protocol started by removing and discarding the culture medium, followed by briefly rinsing the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA

solution to remove traces of serum, which contains trypsin inhibitor. Next, 2 to 3 ml of Trypsin-EDTA solution were added to the flask and the cells were observed under an inverted microscope until the cell layer was dispersed, usually within 5 to 15 min. To avoid clumping, the flask was agitated while waiting for the cells to detach. Flasks where cells were difficult to detach were placed at 37°C to facilitate dispersal. Next, 6.0 to 8.0 ml of complete growth medium were added and cells were aspirated by gentle pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels and incubated at 37°C. Renewal of medium was performed every 2 to 3 days. Cell counts were performed using a hemocytometer. As soon as a full monolayer was observed, cell harvest for control production was performed by removing the culture medium and adding 2 to 3 ml of Trypsin-EDTA solution to the flask, with the cells observed under an inverted microscope until the cell layer was dispersed (usually within 5 to 15 min). Free cells were pipetted to a centrifuge tube and pelleted, after which the medium was removed before DNA or RNA extraction of the cell pellet was performed.

2.5 DNA extraction

DNA was extracted from the pellet of cervical cells using a modified protocol for the QIAamp DNA Mini Kit (Qiagen Inc.). The pellet was resuspended in 180 µl of Buffer ATL and mixed with 20 µl of proteinase K (10 mg/ml) by vortexing. This mixture was incubated for 1 to 2 h at 56°C, after which 200 µl of Buffer AL were added to the mixture. This solution was pulse-vortexed for 15 s to ensure that a homogeneous solution was obtained, which was required for

efficient lysis. The homogeneous solution was incubated at 70°C for 10 min, and the DNA precipitated by the addition of 200 µl of 95% ethanol. The mixture was pulse-vortexed for 15 s and then approximately 600 µl was applied to the QIAamp spin column and centrifuged at 6000 x *g* for 1 min. The extracted DNA was washed by passing 500 µl of Buffer AW1 through the QIAamp spin column at 6000 x *g* for 1 min. The DNA was washed a second time with the addition of 500 µl Buffer AW2 to the column. The wash buffer was passed through the column by centrifugation at 20,000 x *g* for 3 min and then again for 1 min, after which the filtrate was discarded from the collection tube. The QIAamp spin column was placed into a 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was applied to the column. The column was incubated at room temperature for 5 min and then the eluate was collected by centrifugation at 6000 x *g* for 1 min. The eluate was transferred to an appropriately labeled O-ringed screw-cap microcentrifuge tube before storage at -70°C.

2.6 RNA extraction

RNA was extracted from the pellet of cervical cells using a modified protocol for the TRIzol® reagent (Invitrogen). Essentially, cell pellets from aliquots #2 and #3 (see Figure 3) or control material were disrupted in 1 ml of TRIzol with pulse vortexing and then kept at -70°C until queued for extraction. The TRIzol reagent is a monophasic solution of phenol and guanidine isothiocyanate and the extraction protocol for its use is based on the method developed by Chomcynski and Sacchi (Chomczynski & Sacchi, 1987). However, several modifications to the protocol were made in this study to optimize the RNA

recovery while eliminating contamination by DNA. Most of the optimization of the RNA extraction protocol was made with cell-line material, but DNA contamination checks with clinical material prompted one major protocol change and two minor changes during the course of the study. Specimen extractions records were kept to follow which method was applied to each specimen.

The earliest version of the extraction protocol, applied to clinical specimens, was termed version 2 (Appendix C-1). The protocol involved a phase separation, followed by precipitation of the RNA from the aqueous phase. The precipitation was initiated with an equal volume of 100% isopropyl alcohol and facilitated with glycogen. The RNA pellet was washed with 75% ethanol and then air-dried before being dissolved in a 20 µl cocktail containing 2 units of DNase I (Invitrogen), 1X DNase I buffer and 20 units of SUPERase•In™ RNase Inhibitor (Ambion). The reaction was extracted again using TRIzol, followed by the series of steps outlined above, except the final RNA pellet was resuspended in 50 µl of DEPC-treated water. A modification of this protocol involved dissolving the final RNA pellet in 200 µl of DEPC-treated water.

A different version of the extraction protocol termed version 3 (Appendix C-2) was initially applied to clinical specimens and later, with slight modification, became version 4. Version 4 differed from version 3 with the use of 5 units of DNase I instead of 2 units. Essentially, this version involved phase separation followed immediately by another TRIzol extraction of the aqueous phase with identical steps for phase separation and precipitation of the RNA as done in version 2. The DNase I clean-up step was done on the final pellet and then

inactivated using 2 mM EDTA coupled with incubation at 65°C for 10 min. The reaction mixture was then diluted with DEPC-treated water up to 200 µl.

2.7 DNA contamination check and RNA clean-up

The quality and quantity of all RNA preparations were measured before being queued for relative quantification (refer to Figure 3). Quality assessments were done with two primer/probe sets and three real-time assays. The HPV-16 E6 set was used in a real-time PCR assay and with a real-time RT-PCR assay. The level of S9 RNA, as measured by real-time RT-PCR, was used for the overall assessment of the RNA recovery from the cervical cell pellet. Suitable normalization standards are housekeeping genes which maintain steady-state expression across a wide sampling of tumour tissue and normal tissue (Tricarico et al., 2002). Ribosomal protein S9 RNA has been validated for human cervix samples (Erin Travis, BD Biosciences, personal communication). A Ct value in the E6 DNA PCR indicated that DNA contamination was present. A Ct value in E6 RT-PCR, but not in E6 DNA PCR indicated that the RNA preparation was free from DNA contamination and consequently was then queued for relative quantification. A Ct value in E6 DNA PCR queued the RNA preparation for RNA clean-up. No amplification in either E6 RT-PCR or S9 meant that the third aliquot was queued for a repeat attempt at nucleic acid extraction.

Clean-up was performed by treating the RNA preparation with an additional DNase I digestion and, if required, a final clean-up using the RNeasy Mini Kit (Qiagen). DNase treatment was done by adding 11 µl of 10X DNase I Buffer (Invitrogen) to the RNA preparation with 40 units of SUPERase•In™

RNase Inhibitor and 5 units of DNase I, and incubating at 37°C for 30 min. The reaction was stopped by incubating with 12 µl of 25 mM EDTA at 65°C for 10 min. The RNeasy clean-up procedure was done according to the manufacturer's protocol with the optional on-column DNase digestion during RNA purification.

Table 1. Oligonucleotides used for nucleic acid amplification.

Name	Sequence[a]	Final Conc[b]	Ref[c]
Gp5+	TTT GTT ACT GTG GTA GAT ACT AC	0.5	1
Gp6+	GAA AAA TAA ACT GTA AAT CAT ATT C	0.5	1
E1 350L	TRY RKG YYY TAA AAC GAA AGT	0.5	2
E1 547R	TTC CAC TTC AGW AYW GCC ATA	0.5	2
E1 847R	CAA ATC DSW ACA BST KSW TTT ATY RCT YTK AAA	0.25	2
β-ACTIN FORWARD	TCA CCC ACA CTG TGC CCA TCT ACG A	0.3	3
β-ACTIN REVERSE	CAG CGG AAC CGC TCA TTG CCA ATG G	0.3	3
β-ACTIN PROBE	ATG CCC TCC CCC ATG CCA TC	0.2	3
HPV-16 L1 FORWARD	GCT GGT TTG GGC CTG TGT AG	0.3	4
HPV-16 L1 REVERSE	GGC CAC TAA TGC CCA CAC C	0.3	4
HPV-16 L1 PROBE	ATG GCT GAC CAC GAC CTA CCT CAA CA	0.2	4
HPV-16 E6 FORWARD	CTG CAA TGT TTC AGG ACC CA	0.1	3
HPV-16 E6 REVERSE	TCA TGT ATA GTT GTT TGC AGC TCT GT	0.1	3
HPV-16 E6 PROBE	AGG AGC GAC CCG GAA AGT TAC CAC AGT T	0.15	3
HPV-16 E7 FORWARD	AAG TGT GAC TCT ACG CTT CGG TT	0.1	3
HPV-16 E7 REVERSE	GCC CAT TAA CAG GTC TTC CAA A	0.1	3
HPV-16 E7 PROBE	TGC GTA CAA AGC ACA CAC GTA GAC ATT CGT A	0.15	3
HPV-31 E6 FORWARD	AAC CTA CAG ACG CCA TGT	0.1	4
HPV-31 E6 REVERSE	AAT GCC GAG CTT AGT TCA	0.1	4
HPV-31 E6 PROBE	AAT CCT GCA GAA AGA CCT CGG A	0.15	4
HPV-31 E7 FORWARD	GTG TRA GTC TAC ACT TCG TTT G	0.3	4
HPV-31 E7 REVERSE	CAT TAA CAG CTC TTG CAA TA	0.3	4
HPV-31 E7 PROBE	CGA ATA TCT ACT TGT GTG CTC TGT ACA	0.2	4
S9 FORWARD	ATC CGC CAG CGC CAT ATC	0.2	3
S9 REVERSE	TCG ATG TGC TTC TGG GAA TCC	0.2	3
S9 PROBE	AGC AGG TGG TGA ACA TCC CGT CCT T	0.1	3

[a] All probes were labeled with FAM as the reporter dye and with BHQ-1 as the quencher.
[b] micromolar.
[c] 1, de Roda Husman et al., 1995; 2, Ylitalo et al., 1995; 3, Wan-Johanning et al., 2002; 4, This study.

2.8 Oligonucleotides and primer design

All oligonucleotides were synthesized by Sigma-Genosys (Sigma-Aldrich Co.). Primer sequences, final reaction concentrations and references are listed in Table 1. Oligonucleotides for the HPV-16 L1 gene and both HPV-31 targets were designed specifically for this study using Oligo 6 (Molecular Biology Insights, Inc.). Sequence specificity for intended targets was checked using the Basic Local Alignment Search Tool (i.e. BLAST) on GenBank. Furthermore, the potential for mispriming was assessed by inspecting homologous sequence corresponding to the intended primer-annealing site among common mucosal HPV types, including the most phylogenetically related HPV types, using an alignment of DNA sequences. Finally, oligonucleotide sequences were compared with alignments of known sequence variants among the targeted genotype gene to ensure that known nucleotide differences did not lie within primer or probe annealing sites.

2.9 Genotyping by PCR

Preparations of extracted DNA were tested with the hot-start PCR method using either consensus primers targeted for semi-conserved regions within the L1 gene or the E1 gene that generate PCR amplicons of approximately 150 bp and 180 bp respectively. The L1 PCR was used as the primary screening test with negatives queued for testing with the E1 PCR. This redundancy was included to avoid false-negatives due to integration events affecting the HPV L1 gene (Karlsen et al., 1996; Walboomers et al., 1999). The E1 PCR was also used if the quality of the sequence data obtained from the L1 PCR did not meet

the genotyping analysis criteria (see below). The primer sequences for both PCRs and their utility for screening mucosal types of HPV have been published (de Roda Husman et al., 1995; Ylitalo et al., 1995).

The reaction master-mixes for both primer sets had the following similarities; each was adjusted to a final volume of 95 µl using HPLC-grade water and contained 2.5 units of AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1X PCR buffer II (Applied Biosystems), 1.5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and TTP. The L1 PCR reactions contained 0.5 µM of each primer; Gp5+ and Gp6+, whereas E1 PCR reactions contained 0.5 µM of each primer; E1 350L and E1 547R, and 0.25 µM of primer E1 847R. All reactions contained 5 µl of the extracted DNA preparation (quantity unknown). The E1 PCR also contained NP-40 and Tween-20 each at a final v/v concentration of 1%.

2.9.1 Thermal cycling parameters and PCR product detection

PCR amplification was performed with a 9700 thermal cycler (Applied Biosystems). All thermal cycling regimes were preceded by a 10 min, 95°C incubation period, and followed by an additional extension time of 10 min at 72°C and a final soak at 4°C. The reactions were then removed from the thermal cycler and processed, or placed in storage at -20°C. L1 PCRs were cycled 40 times through the following temperature regimes: 94°C for 30 s, 44°C for 60 s, 72°C for 90 s. E1 PCRs were cycled using a two-stage regime with the first stage cycling 15 times through: 94°C for 40 s, 45°C for 40 s, 72°C for 40 s; and

the second stage cycling 30 times through: 94°C for 40 s, 30°C for 40 s, 72°C for 40 s.

Thermal cycled mixtures (20 µl) were resolved electrophoretically on 1.7% agarose gels and visualized under UV light after ethidium bromide staining for fluorescence. Sizes of amplified fragments were estimated by comparison with a 100 bp DNA ladder (Invitrogen). Reactions with visually detectable bands of the appropriate size were queued for DNA sequencing.

2.10 DNA sequencing

2.10.1 Template preparation

The remaining volume of the reaction mixtures for which agarose gel electrophoresis had revealed a single product of expected size, was processed using Microcon® centrifugal filter units (Millipore). The filter units have a 100 kDa nominal molecular weight limit and were used according to the manufacturer's protocol with the nucleic acid recovered from the column using 20 µl of HPLC-grade water.

Purified templates were quantified by visually comparing fluorescence intensity against a mass marker. A portion of the purified templates (4 µl) was resolved electrophoretically on 1.7% agarose gels and visualized by ethidium bromide staining and fluorescence. The quantity of template was estimated by comparison with a Low DNA Mass ladder (Invitrogen).

2.10.2 Cycle sequencing

The purified and quantified PCR products were used in fluorescence-based dideoxy cycle sequencing reactions using the BigDye® Terminator v1.1

Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Essentially, cycle sequencing reaction mixtures of 10 µl final volume contained the following ingredients: 50 ng of template DNA, 4 µl of BigDye terminator premix, and 1.6 pmol of primer. The sequencing primers were the same as those used in the DNA amplification reactions namely; Gp5+, Gp6+ for L1 PCR products or E1 547R for E1 PCR products.

Cycle sequencing reactions were carried out on a 9700 thermal cycler. The sequencing reactions were set up on a cold block, which was kept at 4°C until needed, and placed in the thermal cycler, which was preheated to 96°C. The thermal cycle sequencing regime consisted of 25 cycles of: 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The reactions were held at 4°C until purification.

2.10.3 Purification of sequencing reaction products

Subsequent to cycle sequencing to remove unincorporated dye terminators, the reactions were passed through Centri-Sep™ gel-filtration columns (Princeton Separations) according to the manufacturer's protocol. The purified reaction mixtures were spun in a vacuum centrifuge (Centrivap®, Labconco) at room temperature until dry (typically 10-25 min). The reaction pellets were resuspended in 3 µl of loading buffer [5 parts deionized formamide to one part 50 mg blue dextran / 25 mM EDTA (pH 8.0)].

2.10.4 DNA sequence determination

Automated sequencing was employed using the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems). The resuspended reaction pellets were immediately heat shocked at 95°C for 2 min and then transferred to a Labtop™

cooler (Nalgene) for snap-cooling to -20°C. Samples were then electrophoresed at 100 nucleotides/h with 1680 volts, 150 watts (floating), and 50 mAmp (floating) through denaturing polyacrylamide gels (4% acrylamide, 5% cross-linked, 6 M urea) at a run temperature of 51°C with a gel thickness of 0.2 mm and a well-to-read distance of 36 cm. The fluorescent images were captured with a virtual filter set E and 2400 scans/h using ABI PRISM™ Collection Software v2.6 (Applied Biosystems).

The raw sequencing data were processed using multicomponent analysis, baseline subtraction and scaling with ABI PRISM™ DNA Sequencing Analysis Software v3.4.1 (Applied Biosystems) using the ABI-100 Basecaller module. All base calls were confirmed manually by visual inspection of the electropherograms. The double-stranded DNA sequence information was assembled from consensus L1 sequence data using Autoassembler® v2.1 (Applied Biosystems).

2.11 Genotype analysis

DNA sequence data for either the L1 or E1 region from each HPV positive specimen were independently compared to DNA sequence from known HPV genotypes and confirmed using phylogenetic analysis with a collection of representative mucosal HPV types. The Entrez Nucleotides database was accessed through the internet site maintained by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Regions of local similarity between sequence data from the study specimens and the database were done using the nucleotide-nucleotide BLAST (Altschul et al., 1990). An HPV genotype

determination was made if the BLAST search resulted in a similarity score above 90% and with at least 100 nucleotides of the query providing the score (i.e., dominator ≥100). Indeterminate genotypes were sent to the National Microbiology Laboratory (Health Canada) for resolution using an L1 nested-PCR followed by specialized probe technology using Luminex® coupling beads and flow cytometry, which is well suited for weakly positive samples and mixed-infection determinations respectively (Oh et al., 2007; Schmitt et al., 2006a).

Phylogenetic analysis was performed using BioNumerics v 3.5 (AppliedMaths). DNA sequences were aligned using pairwise comparison with the open-gap penalty set to 100% and unit gap penalty set to 0%. Dendrograms were constructed using the UPGMA clustering method and a grayscale similarity matrix indicating percent sequence identity was used for pairwise comparison as required. Risk categories were assigned based on HPV genotype and previous work indicating odds ratio for cervical cancer associated with the presence of HPV, which was based on pooled data from case-control studies with histologically confirmed squamous-cell cervical cancer and control women (Munoz et al., 2003). In cases of mixed genotypes, the risk category was assigned based on the highest risk genotype.

2.12 Real-time PCR and RT-PCR

The real time RT-PCR conditions used in this study were slightly modified to those used by Wang-Johanning *et al.* (2002). Instead of a two-step RT-PCR, a one-step RT-PCR procedure was used to minimize the possibility of contamination. Quantitect™ Probe PCR and Quantitect™ RT-PCR Probe

reaction kits (Qiagen Inc.) were evaluated with that of alternative suppliers and determined to be the best real-time PCR chemistry kits (data not shown) and were used for real-time PCR and real-time RT-PCR respectively. The Quantitect™ master mix reagent contained HotStarTaq® DNA polymerase. The reverse transcriptase enzymes used with the Quantitect™ RT-PCR Probe reaction kit were packaged separately and 0.25 µl of the reverse transcriptase mixture was added to each RT-PCR reaction immediately before use. The reverse transcriptase enzyme mix contained a proprietary combination of Omniscript™ reverse transcriptase and Sensiscript® reverse transcriptase. The reaction kits contained 5-carboxy-X-rhodamine succinimidyl ester (ROX) for use as an internal reference dye. The ROX dye provided a passive reference signal and was used by the software for the ABI 7700 instrument to automatically normalize the signal of the reporter dye during data analysis, which reduced the non-PCR-related fluorescence fluctuation from well-to-well.

Reaction mixtures contained 12.5 µl of either 2x Quantitect One-Step RT-PCR master mix reagent or 2x Quantitect PCR master mix reagent, 0.25 µl of enzyme mix (RT-PCR only), volume of appropriate oligonucleotide working stock (Table 1), 5 µl of prepared template and HPLC-grade water to make a final volume of 25 µl. The oligonucleotide working stocks consisted of a target-specific primer pair and probe that were mixed in appropriate proportions to give the final concentration as indicated in Table 1 after addition to the reaction mixture.

The real-time RT-PCR thermal cycling consisted of 30 min at 48°C for reverse transcription, 15 min at 95°C to activate the hot-start DNA polymerase, and 40 cycles of 15 s at 95°C, and 60 s at 60°C. The real-time PCR thermal cycling consisted of an initial soak for 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C.

2.13 Data processing

The patient data obtained from the Regina Health District included the scrambled health service number (HSN), date of birth (DOB), date of procedure, accession number, case number, date of test order, case status (i.e., normal or abnormal cervix based on the final clinical diagnosis based on collection of available laboratory test results, patient history and colposcopy findings), Pap result, specimen source for diagnosis text (e.g., cervical biopsy), and diagnosis text (i.e., histology report – freehand pathology observations). Patient age was determined from the difference between DOB and date of procedure. These data and all research data were entered into a relational database (FileMaker Pro 7.0v3). Data were linked together with all test results using the unique identifiers, namely the HSN and study number for patient and study specimen respectively.

The Pap results and histology diagnosis were typically reported using numerical class designations such that squamous intraepithelial lesions (SIL) were classified based on the level of neoplasia observed in the smear or biopsy. Mild dysplasia was categorized as cervical intraepithelial neoplasia 1 (CIN1). Moderate dysplasia was categorized as CIN2 and severe dysplasia as CIN3. The Bethesda System for reporting cervical diagnosis was used infrequently

(n=92 cases) whereby LSIL included HPV associated cellular changes and mild dysplasia, whereas, HSIL included moderate and severe dysplasia. In both Pap classification systems, the term ASCUS was used for cytological findings that did not fulfill the criteria for either benign change or SIL, and can be considered a category for abnormal smear results that were not suitable for grading.

The clinical diagnosis for each patient was classified into one of five categories; abnormal-high, abnormal-medium, abnormal-low, normal or indeterminate. This diagnosis was considered the definitive for comparative purposes and for the diagnostic test evaluations. The diagnosis classification was principally based on histological grading of the cervical biopsies: CIN3, CIN2, CIN1, normal or not done respectively. Moreover, the diagnosis was defined by the collection date of the study sample.

A patient's diagnosis is expected to progress or regress over time and in many cases women have had treatment (i.e., LEEP). Consequently, patient history and/or subsequent histology reports were noted and referred to when needed, but only within the standpoint of the diagnosis at time of study sample collection. Histological diagnoses of HSIL were considered abnormal-high whereas LSIL were considered abnormal-low. A higher grade within a mixed status report moved the status to that grade. For example, a CIN1-2 was considered CIN2 and hence was categorized as abnormal-medium. Likewise, when three biopsies were taken, the status was based on the highest neoplasia found (e.g., if biopsies at 3 o'clock and 6 o'clock were normal but a biopsy at 9

o'clock was found to contain CIN2 then the patient was categorized as abnormal-medium).

Cytological diagnoses were not typically considered in the diagnositic categorization of the patient. On rare occasions, there were cases that had normal biopsies at the time of study sample collection, but with Pap smears classified as abnormal. However, a follow-up visit within 6 months presented an abnormal histology. In such cases, the patient's status was categorized as abnormal despite the normal biopsy at the time the study sample was taken. This was done to reduce incorrect categorization in cases with reasonably apparent but missed histological diagnoses. A recent study showed that one-third of patients that were HPV positive, but with normal histology, were actually false-negative colposcopic biopsy results (Adams et al., 2006).

2.14 Quality assessment

Quality assessments were made on each reaction and on each reaction batch as a whole. All specimen collection and extraction methods and all *in vitro* enzymatic reaction processes on extracted nucleic acids had quality control parameters. For example, each method contained positive and negative controls to ensure that each process performed as expected. Any controls or specimen parameters that produced unexpected results were logged appropriately with corrective action indicated. Good laboratory practice and sound scientific method is implied, but the following text details project-specific control parameters.

Individual amplification plots were examined by visually inspecting each amplification plot for any obvious fluorescence anomalies. The plots were also examined objectively using a statistical method, that detects samples with dissimilar PCR efficiencies. The statistical method, called kinetic outlier detection (KOD) (Bar et al., 2003), required a measured estimation of the PCR efficiency for each replicate reaction. Estimates of PCR efficiency were made using the linear regression of the amplification plot. Linear regression on the logarithmically transformed data of the relative fluorescence per cycle is reported to be an assumption-free method to calculate starting concentrations of mRNAs and PCR efficiencies for each sample (Bar et al., 2003). A computer program, LinRegPCR (Ramakers et al., 2003), was used to perform the calculation on exported raw data from the SDS real-time PCR collection software. KOD was based on the variance of PCR efficiency among three replicates. Outliers, as determined by KOD or detected manually, were then excluded from the average. In this way, the average Ct that was used in subsequent calculations was based only on quality replicates.

Criteria were empirically established to ensure that only quality material was queued for relative quantification. RNA extractions were only analyzed if the preparation had measurable levels of S9 and no signal in the E6 DNA real-time PCR. This quality check was applied again with each relative quantification profile run since the sample RNA was retested for DNA contamination on the same 96-well plate as used in the relative quantification profile run.

After the relative quantification profiling run was complete, the run was categorized as either quality control (QC) failed, QC good+ or QC excellent. These categories were determined using the following criteria; Good+ runs were defined as runs having S9 Ct values in the RNA calibrator controls of <35, whereas excellent runs required S9 levels with control material of <30. QC failed runs had ratios with calibrators <0.70 (calculation coded: R20_R21). Again, KOD ensured that only good replicates were used in averaging. The use of averages helped to eliminate intra-assay variability.

Calibrators were used to control for inter-assay variability. Previously prepared nucleic acids from cell-line material were aliquoted into single-use portions and kept at -70°C until needed. RNA fractions were tested in triplicate using real-time RT-PCR for the same target genes as the sample RNA, namely, transcripts for E6, E7, L1 and S9. DNA fractions of the control material were also tested in triplicate using real-time PCR for E7 and β-actin. It was expected that these calibrators could be used for standardization and thereby control for any run-to-run variations such as differences between in amplification reagent lots, variances in primer/probe working stocks, and any other deviations that may affect reproducibility.

2.15 Relative quantification

Relative quantification (RQ) was based on the relative amount of a target versus a reference by comparing mathematically transformed Ct values. Human β-actin gene was used as the reference for DNA standardizations while transcripts for human ribosomal protein S9 was used as the reference for RNA

standardizations. RQ was used to standardize the amount of HPV DNA to human DNA in the specimen and henceforth will be called viral load. Likewise, RQ was used to standardize the amount of HPV transcript to the human RNA in the specimen. The ratio of standardized RNA to standardized DNA was also calculated to normalize the amount of HPV transcript to the HPV viral load within the specimen. The transformation and comparison of Ct values were done by both using an external standard curve and applying ratios or through a mathematical model as indicated in equation 1 (Pfaffl, 2001). PCR efficiency (E) was either based on theoretically perfect efficiency or estimated. Estimated PCR efficiencies were calculated from either the slope of a standard curve generated using nucleic acid extracted from cell-lines (i.e., $E = 10^{(-1/slope)}$), or from linear regression analysis of each amplification plot as described above.

$$ratio = \frac{(E_{reference})^{Ct_{sample}}}{(E_{target})^{Ct_{sample}}} \div \frac{(E_{reference})^{Ct_{Calibrator}}}{(E_{target})^{Ct_{Calibrator}}} \quad (1)$$

$$concentration = \frac{Ct - 40}{-3.321} \quad (2)$$

These RQ alternatives amount to seven different methods. Method 1 expresses the absolute concentration of HPV-16 E6 transcript levels in the patient sample by converting Ct to concentration using a theoretical external standard curve as indicated in equation 2, where E is assumed to be perfect and -3.321 is used as the slope for the quantification curve. Method 2 is the ratio of HPV-16 E6 transcript to total human RNA as measured by the S9 RT-PCR. Method 3 is the ratio of HPV-16 E6 transcript to total HPV DNA as measured by the HPV-16 E6 DNA PCR. Method 4 is the ratio of the value found in method 2

to a ratio of HPV DNA to total human DNA as measured by the β-actin real-time PCR. Method 5 is the ratio of the value determined in method 4 applied to calibrator RNA and DNA that was run with the sample RNA and DNA. Method 6 is HPV-16 E6 transcript standardized to HPV DNA and Human RNA (i.e., S9 transcript) and Human DNA (i.e., β-actin) and for run-to-run variation using run calibrations that were calculated using equation 1. Method 7 is identical to method 6 except PCR efficiencies were calculated for each reaction using linear regression on the amplification plot (individual PCR efficiency calculation is described in 2.14).

2.16 Statistical analyses

Statistical tests were performed using Prism v4 software (GraphPad). RQ values of all transcripts (i.e., E6, E7 and L1) and HPV DNA regardless of the standardization or normalization approach were sorted by their predetermined diagnostic category. All data sets were analyzed using the D'Agostino-Pearson normality test (omnibus K2) to determine if parametric or nonparametric statistics were appropriate. In any case, nonparametric statistics are more appropriate for the relative quantification data since some values are "off the scale". That is, some transcripts could be considered too low to measure because the RNA levels were below the sensitivity limits of the real-time RT-PCR assay.

The mean or median for each category was calculated for Gaussian or non-Gaussian respectively. The statistical significance of mean or median differences was assessed using either a one-way ANOVA for Gaussian data or the Kruskal-Wallis test for non-Gaussian data. A P-value below 0.05 was

considered statistically significant. For the Gaussian data, the Bartlett's test for equal variances was performed to ensure that a one-way ANOVA was appropriate. To calculate significance between each possible paired category, the Bonferroni or Dunn's post-test were used for Gaussian or non-Gaussian distributions respectively. The value of the test statistic (i.e., Kruskal-Wallis statistic) was used to determine which normalization or standardization of the transcript demonstrated the greatest correlation with the diagnostic categorization.

Contingency tables were constructed to measure the correlation between research data and diagnosis. The statistical significance was tested using the Chi-squared test. If significance was found ($p<0.05$), then grouping was carried out to reduce contingency tables to 2x2 tables. For example, colposcopy diagnoses were categorized into two groups in one of three ways; 1) abnormal-high or abnormal-medium with abnormal-low and normal, 2) abnormal-high with abnormal-medium or abnormal-low with normal, and 3) abnormal-high with abnormal-medium and abnormal-low or normal. The best cutoff for RQ data was determined using ROC curves (Nettleman, 1988). Statistical significance of the 2x2 contingency tables was measured using the Fisher exact test.

The likelihood ratio, sensitivity (test positive / disease present), specificity (test negative / disease absent), positive predictive value (PPV = disease / test positive), and negative predictive value (NPV = non-diseased / test negative) were calculated on all contingency tables. The likelihood ratio (LR) is defined as the probability of the test result for a diseased individual divided by the probability

of the same test result for a non-diseased individual. The positive likelihood ratio is given by LR+ = sensitivity / (1 – specificity) and the negative likelihood ratio is given by LR- = (1 – sensitivity) / specificity. Likelihood ratios do not depend mathematically on prevalence. However, important population characteristics that vary with disease prevalence include the spectrum of disease severity and the referral filter through which patients have passed before the test is applied (Ransohoff & Feinstein, 1978).

A commonly used approach for diagnostic decision making with two dichotomous tests are the 'either positive' rule or the 'both positive' rule. The 'either positive' rule means that the combined test is positive if either component tests are positive. The 'both positive' rule means that the combined test is only positive if both component tests are positive. The utility of the molecular testing in combination with conventional testing with the 'either positive' rule was assessed by comparing likelihood ratios. Comparing the likelihood ratios of two test types can be used to assess the incremental gain from an adjunct test (Macaskill et al., 2002). The combined test is better than either component test when $LR-_{combined} < LR-_{single}$ and $LR+_{combined} > LR+_{single}$. In other words, the combined test must have a higher PPV and NPV than either single test, irrespective of prevalence. The single test is a better choice when $LR-_{combined} > LR-_{single}$ and $LR+_{combined} < LR+_{single}$. That is, the sensitivity of the combined test is always less than the sensitivity of the single test.

Often the choice between using the single or combined test is not clear because there is no simultaneous improvement in both LR+ and LR-. Therefore,

the decision to use the adjunct test is influenced by the trade-off in the expected number of additional false positive results relative to the number of additional true positives. The trade-off will depend on the prevalence of disease and can be calculated using equation 3; where T represents trade-off, R represents the ratio of the number of additional false positives set as acceptable for each additional true positive, Phi represents disease prevalence, D^+ represents the probability for detecting true disease when the combined test is positive while the single component test is negative, D^- represents the probability of a combined test positive with the single component test negative with true non-disease (Macaskill et al., 2002).

$$T = R\Phi D^+ - (1-\Phi)D^- \qquad (3)$$

Additionally, 100T provides an estimate of the trade-off per 100 persons tested whereby T=0 indicates equivalence of the two tests, T > 0 implies that combined test is preferred while T < 0 indicates the prevalence where the single test is preferred. Calculations of 100T against prevalence were plotted for different values of R thereby depicting the expected trade-off per 100 persons tested as R and Φ vary.

The appropriate sample size required to achieve a valid statistical assessment can be calculated before the outset of a study, if the standard deviation of the expected test result is known. This was not possible in this study but the statistical power of the prospective data set was calculated afterward with PS calculator v2.1.3 (Dupont & Plummer, 1998).

3. RESULTS & DISCUSSION

3.1 Epidemiological analysis

The patient data obtained from the Regina Health District, when imported into a relational database, amounted to 4066 entries (i.e. procedures or patient visits). A diagnosis for each patient was obtained corresponding to the collection date of the study sample. The HPV PCR results are presented according to retrospective and prospective portions of the study as indicated in Table 2. In both portions of the study, there were a total of 1369 specimens over a 7-year period (Jan 1998 to Feb 2005) of which 758 specimens were positive for HPV. There was one indeterminate HPV PCR result from a specimen that was inadvertently left for three days at RT for which a false-negative could not be ruled out. The proportion of positive results did not differ significantly from retrospective to prospective portions of the study. The average HPV prevalence was 55.4% and ranged from 47.0% in 1998 to 65.4% in 1999. It appeared that the HPV detection rate was not affected by the difference in sampling strategy between the retrospective and prospective portions of the study.

The women aged 35 years or younger presented with a higher percentage of HPV infection (70%) than the women over 35 years of age (30%). This was not unexpected. A similar study found that 85% of women ≤ 35 years of age who were referred to a cervical pathology clinic were HPV positive, while 54% of women over 35 years of age were HPV positive (Gonzalez-Bosquet et al., 2006).

Table 2. HPV prevalence among study specimens obtained from women referred for colposcopy clinic at the Regina General Hospital, Saskatchewan.

Study	PCR Result	Total
Retrospective	Negative	260
	Positive (55.5%)	324
	Retrospective Total	584
Prospective	Indeterminate	1
	Negative	350
	Positive (55.3%)	434
	Prospective Total	785
	Grand Total	1369

This table demonstrates that despite differences in the recruitment strategy between the two study periods the prevalence of HPV was similar.

The percentage of women with a positive result for HPV with an abnormal histology of CIN1, CIN2 or CIN3 was 34%, 20% and 28% respectively with the remaining 18% of the HPV positive women with normal histology. The percentage women with HPV having normal histology is not entirely unexpected since HPV DNA testing is better correlated with infection than histology. One likely explanation is that the biopsy procedure missed any tissue abnormality that may have been present. It is uncommon for the cytology to revert to normal before the viral infection has cleared (Syrjanen et al., 2005). On the contary, 51.2% of women that were HPV negative had an abnormal histology at the time the study sample was taken. Histology is generally considered correct and was regarded here as the definitive reference test (Roteli-Martin et al., 2001). Consequently, any patient with abnormal histology that was HPV DNA negative was considered a false-negative PCR result and was reflected in a poor test sensitivity (refer to section 3.2.2). However, there are no evidence to suggest that such false negatives were biased to any particular HPV type (refer to 3.1.2) and were rather likely missed due to inadequate collection of infected cells for DNA extraction or at least resulted in HPV DNA recovery that was below the limit of detection for the HPV DNA PCR test. It is important to consider that although the use of colposcopy-directed biopsies is commonly considered an appropriate reference it also has limitations. Recent findings suggest that colposcopy-directed biopsy, as used here, has a sensitivity of 74.7% (Pretorius et al., 2007).

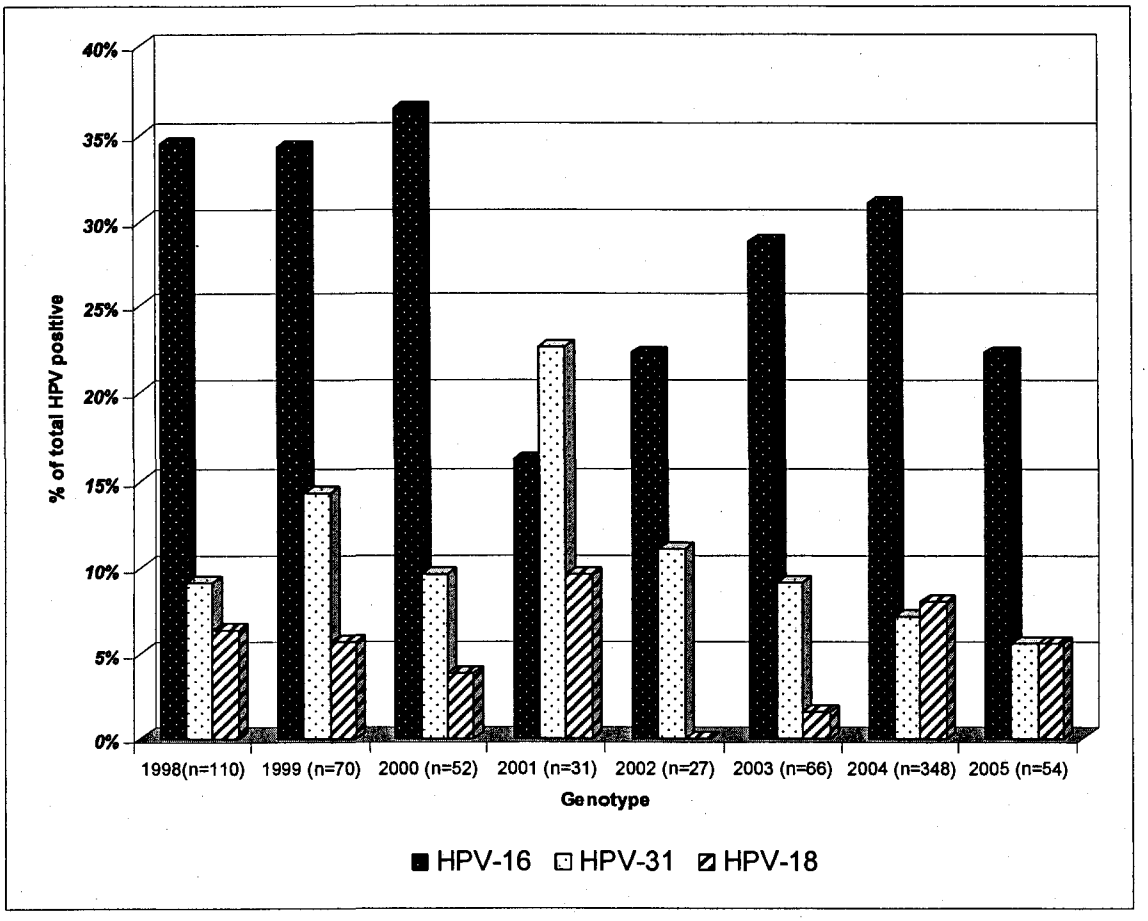


Figure 4. Percentage of the most commonly observed HPV genotypes among HPV DNA positive samples collected from women referred to colopscopy by year.

For this reason, women were categoried by final diagnosis of the physican, which are dependent on all screening test results, and colposopy findings (i.e. normal, abnormal-low, abnormal-medium and abnormal-high).

3.1.1 HPV distribution

During the seven-year collection period, 1355 DNA samples were matched to 1166 patients, of which 858 patients had histological data (linked to 927 DNA samples) and 655 patients had Pap testing done at the time the DNA sample was taken (linked to 685 DNA samples). The prevalence of HPV infection with each diagnosis category is indicated in Table 3. Basically, the 927 DNA samples that were matched to patients with histological data were diagnosed as follows: 177 (19.0%) were abnormal-high, 129 (13.9%) were abnormal-medium, 344 (37.1%) were abnormal-low and 278 (30.0%) had a normal diagnosis at the time the study specimen was taken. Seven hundred and fifty-three (55.6%) of the samples collected from women at the clinic were positive for HPV DNA. The most commonly identified genotype was HPV-16 (18.2%) followed by HPV-31 (6.1%) and then HPV-18 (3.6%). Fifteen specimens were co-infected with HPV types 16 and 31. Figure 4 shows the distribution of these top three genotypes by year of collection. Table 4 shows the distribution of the four most common HPV types among all women referred to colposcopy and within two subpopulations of those women: those with any of the fifteen high-risk HPV types and those having histology of CIN2 or greater.

Table 3. Prevalence of HPV Infections at a colposcopy clinic in Regina, SK.

	Number (%) of specimens with										
HPV Status	Normal		Abnormal-high		Abnormal-med		Abnormal-low		Ind	Total	
Positive	101	(36.3)	159*	(89.8)	112	(86.8)	192	(55.8)	189	753	(55.6)
Single	93	(33.5)	145*	(81.9)	101	(78.3)	174	(50.6)	166	679	(50.1)
Double	1	(0.4)	7	(4.0)	7	(5.4)	5	(1.5)	7	27	(2.0)
Untypeable	7	(2.5)	7	(4.0)	4	(3.1)	13	(3.8)	16	47	(3.5)
Types(a)											
HPV-6	2	(0.7)	0		4	(3.1)	8	(2.3)	3	17	(1.3)
HPV-11	2	(0.7)	0		1	(0.8)	5	(1.5)	5	13	(1.0)
HPV-16	24	(8.6)	93	(52.5)	50	(38.8)	49	(14.2)	31	247	(18.2)
HPV-18	12	(4.3)	7	(4.0)	5	(3.9)	15	(4.4)	10	49	(3.6)
HPV-30	0		0		0		0		1	1	(0.1)
HPV-31	6	(2.2)	24	(13.6)	21	(16.3)	17	(4.9)	15	83	(6.1)
HPV-32	2	(0.7)	0		0		0		1	3	(0.2)
HPV-33	3	(1.1)	10	(5.6)	7	(5.4)	7	(2.0)	5	32	(2.4)
HPV-34	0		0		0		0		1	1	(0.1)
HPV-35	4	(1.4)	2	(1.1)	4	(3.1)	5	(1.5)	13	28	(2.4)
HPV-39	4	(1.4)	2	(1.1)	0		4	(1.2)	3	13	(1.1)
HPV-40	0		0		0		1	(0.3)	2	3	(0.3)
HPV-42	2	(0.7)	0		0		5	(1.5)	6	13	(1.1)
HPV-44	1	(0.4)	1	(0.6)	0		1	(0.3)	2	5	(0.4)
HPV-45	3	(1.1)	3	(1.7)	3	(2.3)	3	(0.9)	2	14	(1.2)
HPV-51	0		1	(0.6)	2	(1.6)	0		3	6	(0.5)
HPV-52	1	(0.4)	5	(2.8)	2	(1.6)	0		5	13	(1.1)
HPV-53	0		0		1	(0.8)	1	(0.3)	3	5	(0.4)
HPV-54	0		0		0		0		2	2	(0.2)
HPV-55	2	(0.7)	0		0		0		2	4	(0.3)
HPV-56	6	(2.2)	1	(0.6)	2	(1.6)	15	(4.4)	9	33	(2.8)
HPV-58	4	(1.4)	3	(1.7)	2	(1.6)	8	(2.3)	7	24	(2.0)
HPV-59	0		2	(1.1)	4	(3.1)	4	(1.2)	14	24	(2.0)
HPV-61	0		0		0		0		1	1	(0.1)
HPV-62	1	(0.4)	0		0		1	(0.3)	4	6	(0.5)
HPV-66	3	(1.1)	0		1	(0.8)	3	(0.9)	3	10	(0.8)
HPV-67	2	(0.7)	1	(0.6)	0		5	(1.5)	4	12	(1.0)
HPV-68	0		1	(0.6)	0		0		1	2	(0.2)
HPV-70	0		0		1	(0.8)	2	(0.6)	1	4	(0.3)
HPV-73	2	(0.7)	2*	(1.1)	3	(2.3)	7	(2.0)	6	20	(1.7)
HPV-74	1	(0.4)	0		0		2	(0.6)	4	7	(0.6)
HPV-81	0		0		1	(0.8)	4	(1.2)	2	7	(0.6)
HPV-82	1	(0.4)	1	(0.6)	0		1	(0.3)	1	4	(0.3)
HPV-83	0		0		0		3	(0.9)	0	3	(0.3)
HPV-84	0		0		0		1	(0.3)	2	3	(0.3)
HPV-86	2	(0.7)	0		0		0		1	3	(0.3)
HPV-87	1	(0.4)	0		0		2	(0.6)	2	5	(0.4)
HPV-90	4	(1.4)	1	(0.6)	1	(0.8)	4	(1.2)	2	12	(1.0)
HPV-91	0		0		1	(0.8)	2	(0.6)	3	6	(0.5)
Negative	177	(63.7)	17	(9.6)	17	(13.2)	152	(44.2)	239	602	(44.4)
Total	278		177		129		344		428	1355	

* includes one sample of vulvar carcinoma in situ

(a) regardless of whether single or double

Table 4. Percentage of the most common HPV types among patient category within women referred for colposcopy at the Regina General Hospital.

HPV type	Category		
	% of women at Clinic	% of women with High-risk HPV	% of women with ≥CIN2
HPV-16	18.2	41.1	46.7
HPV-31	6.1	13.8	14.7
HPV-18	3.6	8.2	3.9
HPV-56	2.8	5.5	0.7

The high prevalence of HPV-31 is distinctive since the pattern in most parts of the world, in descending order of frequency, consists of types HPV-16, HPV-18, HPV-45 and HPV-31 as the most common types in both women with cytologically normal cervixes and women with invasive cervical cancer (Hindryckx et al., 2006; Kjaer et al., 2006; Munoz et al., 2003). Interestingly, a recent HPV testing proficiency survey of 29 laboratories in 12 countries found that HPV 31 was the least accurately detected by participating laboratories (Quint et al., 2006). However, there is no evidence that the HPV typing protocol used in this study would be better than others for HPV-31 detection since the same consensus primers set (i.e. Gp5+ and Gp6+) were used as in other studies. In addition, HPV-31 was more prevalent in both retrospective and prospective data sets which used a different screening approach for HPV typing.

To ensure that no biases were introduced into the HPV genotype prevalence assessment with the L1 consensus primers, all negative specimens were tested with a second PCR, which targeted the E1 ORF (Ylitalo et al., 1995). This redundancy has been recommended to avoid false-negatives due to integration events that may disrupt the HPV L1 gene (Karlsen et al., 1996; Walboomers et al., 1999). In addition, 41 indeterminate genotypes from 2001 to 2005 were reassessed using qualitative judgment of PCR product quantity and sequence quality by visually inspecting the gel photos and electropherograms. Nine of the 41 indeterminate samples had sufficient PCR product, but had poor sequence quality, as indicated with peak-under-peak noise. Luminex® technology on a nested L1 PCR found 8/41 (19.5%) samples to be mixed HPV

infections, which is at a considerably higher prevalence than the overall 3.5% found with the DNA sequencing protocol. All resolved HPV infections with the Luminex method have been included in Table 3. The number of infection with multiple HPV types is therefore underestimated in this study, but the relative frequencies of the most common types, HPV-16, HPV-18 and HPV-31, should not be significantly biased. Mixed infection with HPV-31 and HPV-16 were confirmed with type specific PCR primers.

3.1.2 Test performance for correct dichotomization

The results of the statistical analyses of the testing methods for the correct separation of patients according to histologically based clinical diagnosis after colposcopy are summarized in Table 5. Analyses of the Pap test were restricted to 685 specimens, which had useable data and were classified using CIN nomenclature. Samples were excluded from the examination of Pap performance if they were either ASCUS (n=107), not done (n=39) or graded as HSIL or LSIL (n=92). Four other samples were excluded because the Pap results simply indicated "HPV associated cell changes were present". In a similar fashion, indeterminate HPV typing results were not included in the contingency tables used for statistical analyses and consequently did not affect the estimates of test performance indicators shown in Table 5.

Table 5. Summary of dichotomization performance by test for the correct categorization of women referred for colposcopy.

Data set	Grouping[a]	P	Likelihood	Sens	Spec	PPV	NPV
Pap	High vs Med-Normal	<0.0001	8.14	0.55	0.96	0.75	0.91
	High-Med vs Low-Normal	<0.0001	15.3	0.58	0.96	0.87	0.84
	High-Low vs Normal	<0.0001	6.80	0.57	0.92	0.93	0.51
PCR	High vs Med-Normal	<0.0001	1.68	0.90	0.46	0.28	0.95
	High-Med vs Low-Normal	<0.0001	1.89	0.89	0.53	0.48	0.91
	High-Low vs Normal	<0.0001	1.96	0.71	0.64	0.82	0.49
Genotype	High vs Med-Normal	<0.0001	1.13	1.00	0.12	0.33	1.00
Risk	High-Med vs Low-Normal	<0.0001	1.13	0.97	0.14	0.55	0.83
	High-Low vs Normal	0.8201	NA	NA	NA	NA	NA

[a] High, Med and Low are abbreviations for abnormal-high, abnormal-medium and abnormal-low respectively, which were generally based on histological diagnosis of CIN3, CIN2 and CIN1.

Detection of HPV DNA increases the likelihood of an abnormal diagnosis by almost twofold (Table 5, PCR set, High-Low vs Normal grouping). However, the sensitivity of HPV DNA for the detection of an abnormal cytology is only 0.71 with a poor negative predictive value (NPV) of only 0.49. On the other hand, the sensitivity of the HPV DNA testing algorithm used here for the detection of HSIL was 0.90 and is better than most published sensitivities, which range from 0.68 to 0.98 and an average 0.85 (Ghim et al., 2002). An abnormal Pap of any grade is 93% accurate (within the colposcopy clinic) in the detection of histologically confirmed dysplasia whereas a normal Pap needs to be followed carefully. Consequently, the detection HPV DNA in a primary screen will require the development of guidelines for appropriate management and must include negative and borderline cytology. This incorporates the high sensitivity of HPV screen for detection HSIL along with the high specificity of Pap test for detection of abnormal histology.

That said, taking HPV DNA testing beyond simple detection to typing provides valuable information. In 2001, HPV-31 was the most common HPV infection in women that were referred for colposcopy (Figure 4), although this figure may not be significant due to the low number (n= 31) collected in that year. The prevalence of HPV-31 has been consistently the second highest to HPV-16 and only matched by HPV-18 in 2004 and 2005.

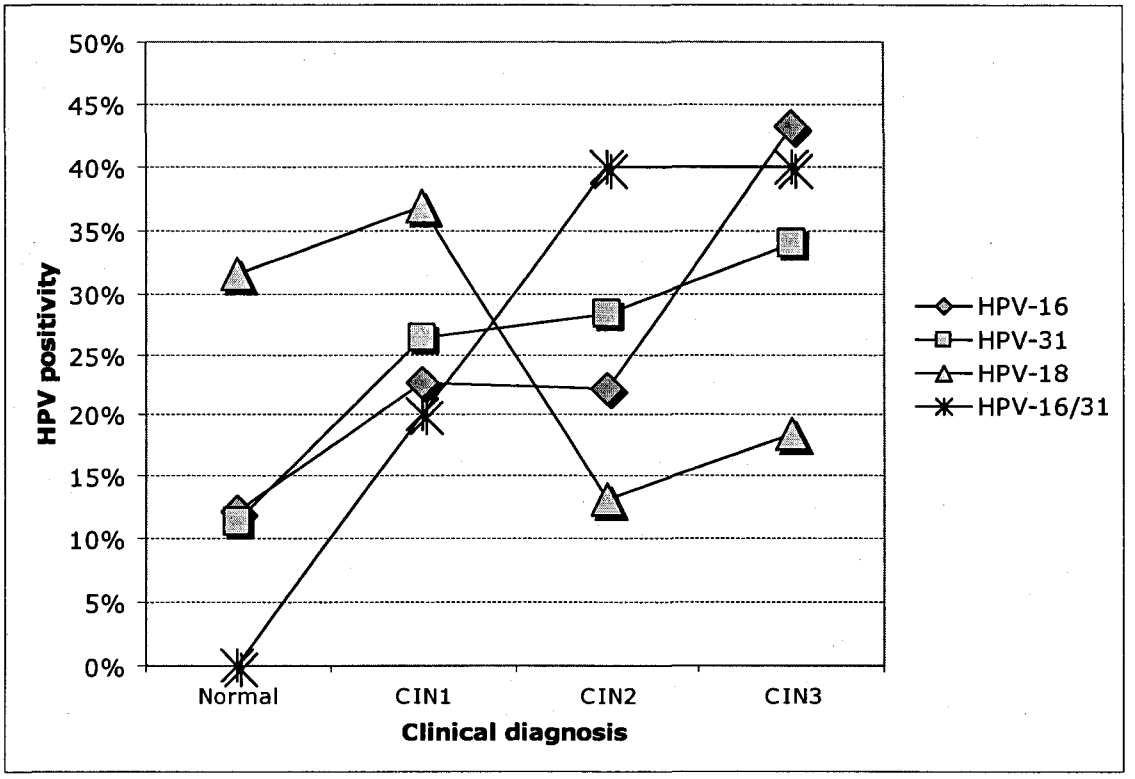


Figure 5. Most common HPV types compared across clinical diagnosis.

Inspection of the shape for the diagnosis curve for the three most common HPV genotypes (Figure 5) reveals that HPV-16 and HPV-31 share a striking similarity, whereas HPV-18 is much different. This difference in distribution of diagnosis is statistically significant (P = 0.0039). The Fisher exact test on contingency tables from pairwise comparisons substantiate the difference between HPV-18 and the other two more common genotypes (Table 6). Furthermore, the risk of cervical disease associated with HPV-18 infection is substantially lower than for either HPV-16 or HPV-31. Table 7 shows for the three most common HPV types among women referred to colposcopy the odds ratio for an abnormal histology of any grade (Normal vs. CIN1-3), the odds ratio for an abnormal histology of CIN2 or CIN3 (Normal vs. ≥CIN2) and the odds ratio for CIN3 (Normal vs. ≥CIN3).

Statistical testing supports the observation that patients with a diagnosis of ≥ CIN2 are more likely to be infected with HPV-16 or HPV-31 than HPV-18. Infection with HPV-16 and HPV-31 increases the likelihood of a histological diagnosis of CIN2 or greater. Consequently, in Saskatchewan's population, not only is the relative prevalence of HPV-18 lower than most worldwide prevalence rates, but also an infection with HPV-18 appears to be relatively more benign. Therefore, HPV-16 and HPV-31 manifest either more virulent or more persistent infections in our population. A higher prevalence of HPV-31 types has been observed in other populations as well (Beerens et al., 2005; Hindryckx et al., 2006; Rassu et al., 2005). This information should be considered in the design of HPV detection

Table 6. Relative risk assessment of CIN with HPV types 16, 18 and 31 for women referred for colposcopy.

	≥CIN2 vs ≤CIN1					
	P	Likelihood	Sensitivity	Specificity	PPV	NPV
31 vs 18	0.0056	1.687	0.73	0.56	0.62	0.68
16 vs 18	0.0002	1.260	0.92	0.27	0.65	0.68
16 vs 31	0.7468	1.029	NA	NA	NA	NA

Table 7. Odds ratios for cervical disease in women referred for colposcopy and habouring HPV types 16, 18 and 31.

	Odds ratio (95% CI)[a]		
	Normal vs Abnormal	Normal vs ≥ CIN2	Normal vs ≥ CIN3
HPV-16	6.3 (3.6-11.0)	3.6 (2.1-6.3)	4.5 (2.5-8.2)
HPV-31	4.3 (1.8-12.6)	3.2 (1.3-9.3)	2.8 (1.1-8.7)
HPV-16/31	11.42 (1.3- ∞)	9.8 (1.0- ∞)	8.7 (0.8- ∞)
HPV-18	0.8 (0.4-1.7)	0.3 (0.1-1.0)	0.3 (0.1-0.9)

[a] calculated by the Mantel-Haenzel test

methods and the development of vaccines for the prevention of cervical cancer. The higher likelihoods for disease with HPV-31 could have implications for the triage policy of women within Saskatchewan.

3.1.3 Monitoring vaccine efficacy

Impact studies of the new HPV vaccines will be biased unless local base-line distribution studies are conducted. Vaccine cross-protection for other important oncogenic HPV types and the emergence of potential genotype replacements require the knowledge of the pre-vaccine epidemiology of HPV. Certainly, the distribution of HPV-31 should be specifically examined in other populations which currently only categorize it with other high-risk types. One could speculate that HPV-31 may become the most prevalent high-risk HPV type after the HPV vaccine becomes widely used within the Saskatchewan population, and perhaps others.

In this study, a base-line of the molecular epidemiology of HPV in a population of women attending a colposcopy clinic in Regina, Saskatchewan, was obtained by typing 1355 specimens collected over a period of 7 years. The overall HPV positivity rate was 50.1%. As noted above, the type distribution showed a predominance of HPV-16 (18.2%), as expected, but the second most common type was HPV-31, with a prevalence of 6.1%. HPV-18 was third with a prevalence of 3.6%. The prevalence of each specific type fluctuated over the years and, in 2001, HPV-31 was the most common HPV infection in women that were referred for colposcopy (Figure 4). This observation was unexpected as most studies have found HPV-18 as the second most prevalent high-risk HPV

type in clinical populations (Dunne et al., 2007; Munoz et al., 2003; Weller & Stanberry, 2007). However, a higher prevalence of HPV-31 types has been observed in some populations (Beerens et al., 2005; Hindryckx et al., 2006; Rassu et al., 2005), and studies on European populations have also shown HPV-31 as the second most common HPV type in low-grade cervical lesions (Clifford et al., 2005b). Other studies, especially in Asia, have found HPV-58 or HPV-52 as the second most common type (Camara et al., 2003; Lin et al., 2006).

It is becoming apparent that each jurisdiction will need the knowledge of the base-line of HPV causing disease in their community. This study, as well as others (Beerens et al., 2005; Hindryckx et al., 2006; Rassu et al., 2005), have identified a relatively high prevalence of HPV-31 in certain populations. Base-line studies will be compromised if HPV is only categorized as either high-risk or low-risk without discriminating as to which actual HPV types are present. DNA sequencing is insufficient in identifying all mixed high-risk types. Hence, the necessary quality assurance needs to be in place to ensure sensitivity to all high-risk types. In populations where HPV-31 is already significant it has the potential to become the most prevalent high-risk HPV under vaccine selection. Monitoring systems will need to have the ability to track cross-protection to accurately predict the local impact of cervical cancer vaccines.

In order to ensure an unbiased detection of HPV types this study used redundant detection systems (L1 PCR and E1). It is known that L1 PCR alone (using Gp5+ & Gp6+ primers) could miss certain types, e.g., HPV-52 (Chan et al., 2006a), and redundancy has been recommended to avoid false-negatives

due to integration events affecting the HPV L1 gene (Karlsen et al., 1996; Wang-Johanning et al., 2002). Consequently, to ensure that no biases were introduced into the HPV genotype prevalence assessment all negatives with the L1 consensus primers were queued for a second PCR, which targeted the E1 ORF (Ylitalo et al., 1995). This study also employed specific primers for HPV-16 and HPV-31 to determine the exact frequencies of these types. In fact, the true prevalence of HPV-31 in other populations may be underestimated. A recent study has show that half of 29 laboratories in 12 countries using a variety of methods failed to detect high concentrations of HPV-31 with a proficiency panel making it the least accurately detected HPV type (Quint et al., 2006). In this study, the number of infections with multiple HPV types is likely to be underestimated but the relative frequencies of the most common types, HPV-16 and HPV-31, should not be significantly biased. The frequency of co-infection and the ability of HPV to recombine (Angulo & Carvajal Rodriguez, 2007) will be an important factor that may drive genotype replacement after HPV vaccination is implemented. It was shown here that the occurrence of mixed infections with HPV-16 and HPV-31 is more common than traditional typing methods have typically indicated (Munoz et al., 2003).

In conclusion, the Saskatchewan population shows a higher than expected prevalence of HPV-31 associated with high-grade lesions. In contrast, the prevalence of HPV-18 is lower than most worldwide prevalence rates and an infection with HPV-18 appears to be relatively more benign. This information should be considered in the design of HPV screening methods and the

assessment of cost effectiveness of the current type-specific vaccines which has been estimated using type-specific prevalence data from selected populations including those outside the North America (Villa, 2006; Villa et al., 2006). The base-line prevalence data in this study can be used as a denominator for post-vaccine surveillance. Moreover, regular re-sampling of patients referred to colposcopy will identify the effect of vaccination on the burden of cervical disease, HPV-16 and HPV-18 infection, cross-protection or increased prevalence of other HPV types. In particular, it will be interesting to note the burden of disease caused by high-grade lesions associated with HPV-31 after the implementation of HPV vaccines.

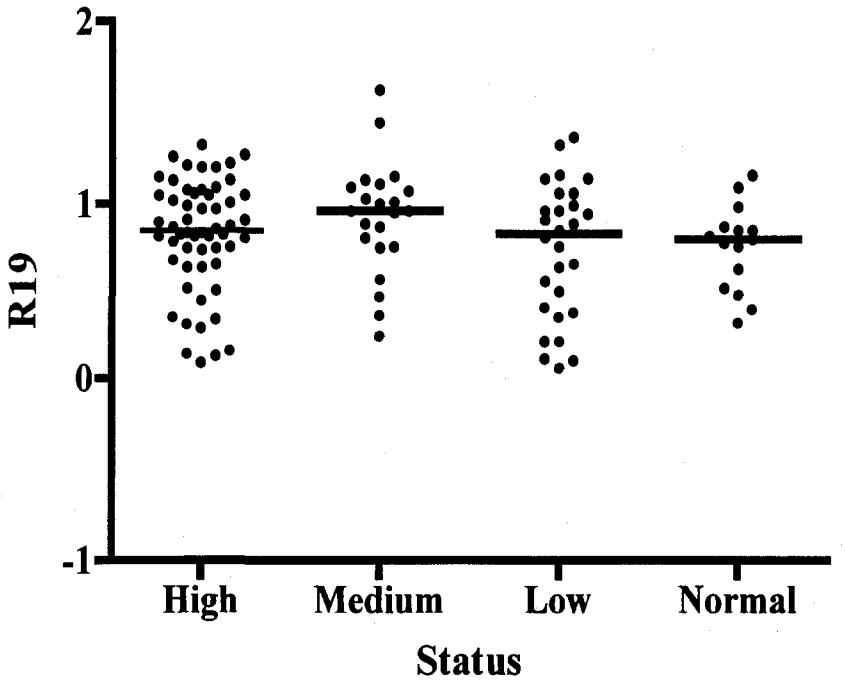


Figure 6. Correlation between HPV viral load and clinical diagnosis.

R19 is the ratio of HPV DNA levels to Human DNA levels in sample. DNA levels are based on calculated concentrations using a hypothetical perfect standard curve. Status refers the clinical diagnosis of the patients cervix at the time of study sample collection. Each sample is indicated with a dot while the median for each group is indicated with a solid line.

3.2 HPV-16 quantiation analyses

3.2.1 Viral load analysis

The viral load data are Gaussian as supported by the D'Agostino-Pearson normality test. The Bartlett's test gave a P value of 0.2887 indicating that the group variances were not significantly different. Likewise, ANOVA gave a P value of 0.2075 indicating that the mean values for each group were not statistically significantly different. Figure 6 displays the range and median of HPV viral load in the samples. Even visually, it's apparent that there is no difference among the level of HPV DNA between diagnosis categories.

It appeared that with HPV-16 positive women who have been referred to colposcopy that there is no reliable difference among the level of HPV DNA between diagnosis categories. This finding is in agreement with several other studies that have found a very poor predictive value for HPV viral load or its correlation with cervical abnormalities (Andersson et al., 2006; Chan et al., 2005; Monnier-Benoit et al., 2006; Ordi et al., 2005; Wensveen et al., 2005). Other studies that have found weakly supported viral load differences have used nonparametric statistical tests on a Gaussian distribution (Carcopino et al., 2006; Snijders et al., 2006a), a procedure that tends to artifactually reduce P values. Other studies state there is a significant difference only when comparing HPV viral load to that of HPV-negative women, which confirms that HPV positivity and not viral load is the significant factor (Flores et al., 2006). Intuitively, it makes sense that HPV viral load is predictive of lesion size rather than dysplasia grade and this has been found to be the case (Sun et al., 2001).

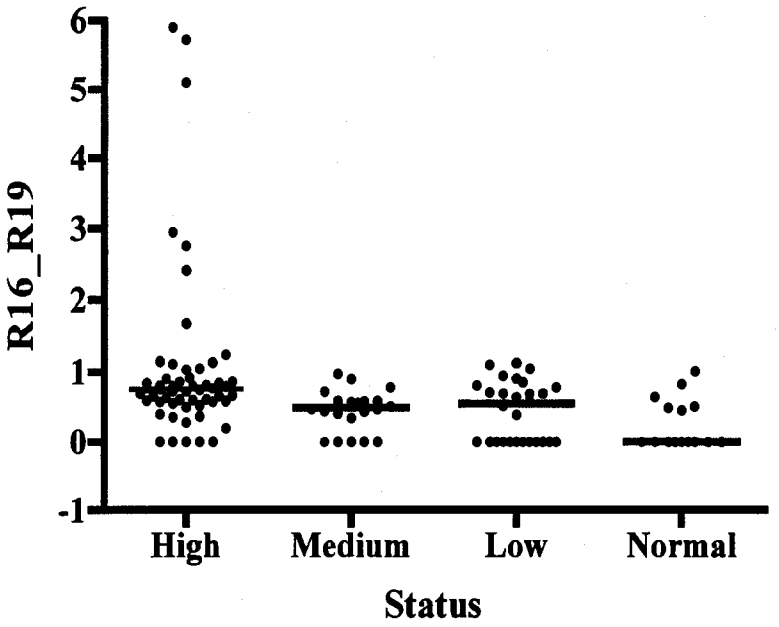


Figure 7. Correlation between the relative E6 transcript quantity, corrected for total RNA & DNA and viral load, and clinical diagnosis.

R16_R19 represents the RQ of HPV-16 E6 transcript. Transcript levels are relative to total amounts of RNA and DNA in the sample and have been standardized for HPV viral load. RQ values were based on mathematically transformed Ct values that were collected using real-time PCR. Status refers the diagnosis at the time of study sample collection. Each sample is indicated with a dot while the median for each group is indicated with a solid line.

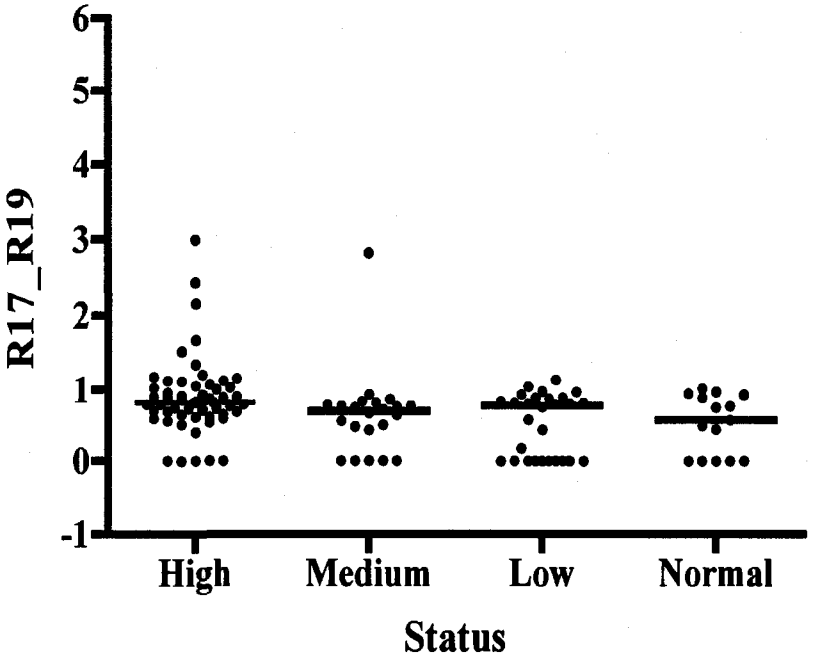


Figure 8. Correlation between relative E7 transcript quantity, corrected for total RNA & DNA and viral load, and clinical diagnosis.

R17_R19 represents the RQ of HPV-16 E7 transcript. Transcript levels are relative to total amounts of RNA and DNA in the sample and have been standardized for HPV viral load. RQ values were based on mathematically transformed Ct values that were collected using real-time PCR. Status refers the diagnosis at the time of study sample collection. Each sample is indicated with a dot while the median for each group is indicated with a solid line.

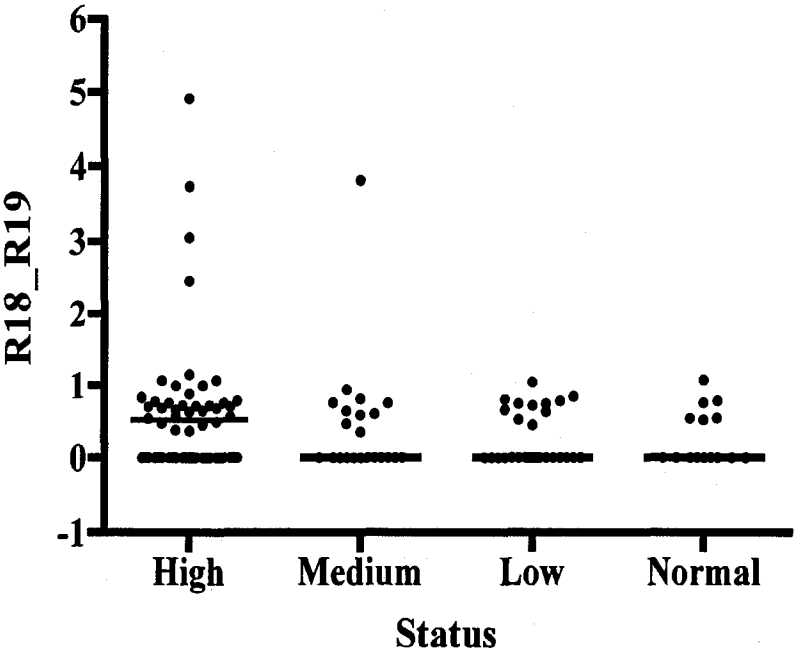


Figure 9. Correlation between relative L1 transcript quantity, corrected for total RNA & DNA and viral load, and clinical diagnosis.

R18_R19 represents the RQ of HPV-16 L1 transcript. Transcript levels were relative to total amounts of RNA and DNA in the sample and standardized for HPV viral load. RQ values were based on mathematically transformed Ct values that were collected using real-time PCR. Status refers the diagnosis at the time of study sample collection. Each sample is indicated with a dot while the median for each group is indicated with a solid line.

3.2.2 Relative quantification of HPV transcripts for cervical diagnosis

Figures 7, 8 and 9 show RQ of HPV-16 transcripts E6, E7 and L1 respectively as calculated by method 4 (see 2.15 and 3.3.7). Normality testing confirmed that RQ of the HPV transcripts produced data that were non-Gaussian. The presence of E6 transcripts (Chi-squared, $P = 0.0002$) and the level of E6 gene transcription ($P < 0.0001$, KS = 22.77) relative to viral load and host nucleic acid (i.e. E6 RQ) emerge to be the most statistically significant finding, with respect to correlation with cervical abnormalities. The Dunn's multiple comparison tests showed that the median level of the E6 transcript was significantly different for the following pairs: abnormal-high vs abnormal-medium ($P < 0.01$), abnormal-high vs abnormal-low ($P < 0.05$) and abnormal-high vs normal ($P < 0.001$). E7 levels were also statistically significant ($P = 0.0096$, KS = 11.43) but, unlike E6, the post-test revealed no statistical significance between groups of cytological abnormalities. For L1 transcripts, no statistical significance for either its presence (Chi-squared, $P = 0.2357$) or its transcription levels ($P = 0.2454$, KS = 4.153) were found. Essentially, L1 transcripts have no correlation with the transformed state with either its presence, or its level (i.e. RQ) when present.

3.2.3 Diagnostic performance evaluation

The samples for repeat Pap testing were taken first, followed by a brush for HPV nucleic acid extractions and finally a biopsy for histology. Therefore, the performance of these tests should be considered as they might perform as a

triage tool before the scheduled colposcopy. The study population comprised of women who had two previously abnormal Pap test results within six months or those who had a single Pap test result of ≥ CIN3. In total, 887 samples were collected during the study period. All samples were tested for HPV DNA and genotyped if positive. The results from these tests are tabulated in Tables 8, 9 and 10. One hundred and twenty-one samples that were HPV-16 positive were queued for RNA analysis. The results of HPV RNA detection of E6 transcripts are tabulated in Table 10. The results of RQ of E6 transcripts are tabulated in Table 11.

Table 12 shows the sensitivity, specificity and likelihood ratios for three diagnostic tests and combinations of them: Pap testing, HPV DNA detection and genotyping. No single test demonstrates superiority in both sensitivity and specificity. Pap testing is more specific for the detection of any abnormality (i.e. ≥ CIN1) while genotyping had the best specificity for disease of CIN2 or greater. Of course the idea of triage is to get the most women affected with disease referred while implementing stringent monitoring for women with low-grade disease and eliminating women from triage with normal cervixes. With this objective, a combination of tests using an 'either positive' rule will improve sensitivity and capture the most number of women with disease. The evaluation here was to measure the impact that combination testing would have on both sensitivity and specificity and the implications of a cascade testing strategy for triage.

Table 8. Stratification of women by Pap and HPV DNA test results with categories of cervical disease (n=887).

Pap result	Disease Category[a]					
	Normal		Abnormal Low		Abnormal Med & High	
	HPV DNA		HPV DNA		HPV DNA	
	positive	negative	positive	negative	positive	negative
HSIL	5	2	13	2	156	7
LSIL	14	8	84	24	51	9
ASCUS	20	19	30	21	17	3
Normal	59	147	58	97	30	11
Total	98	176	185	144	254	30

[a] Largely derived from histological grading of biopsies obtained during colposcopic examinations with CIN1, CIN2 and CIN3 corresponding to abnormal-low, abnormal-med and abnormal-high respectively.

Table 9. Stratification of women by Pap and HPV risk specific genotype result with categories of cervical disease (n=887).

Pap result	Disease Category[a]					
	Normal		Abnormal Low		Abnormal Med & High	
	Genotype Risk[b]		Genotype Risk		Genotype Risk	
	High	Low	High	Low	High	Low
HSIL	5	2	13	2	147	16
LSIL	14	8	68	40	47	13
ASCUS	17	22	21	30	14	6
Normal	50	156	44	111	30	11
Total	86	188	146	183	238	46

[a] Largely derived from histological grading of biopsies obtained during colposcopic examinations with CIN1, CIN2 and CIN3 corresponding to abnormal-low, abnormal-med and abnormal-high respectively.

[b] Genotype information was used to categorized women based on published risk classification (Munoz et al., 2003). High risk category included probable high-risk types HPV-23, HPV-53 and HPV-66 and unclassified HPV types. Low-risk included HPV DNA negative women.

Table 10. Stratification of HPV-16 positive women by Pap and HPV E6 RNA with categories of cervical disease (n=121).

Pap result	Disease Category[a]					
	Normal		Abnormal Low		Abnormal Med & High	
	HPV E6 RNA		HPV E6 RNA		HPV E6 RNA	
	positive	negative	positive	negative	positive	negative
HSIL	0	1	1	1	50	4
LSIL	2	1	10	4	14	1
ASCUS	0	1	1	1	2	0
Normal	4	5	4	6	2	6
Total	6	8	16	12	68	11

[a] Largely derived from histological grading of biopsies obtained during colposcopic examinations with CIN1, CIN2 and CIN3 corresponding to abnormal-low, abnormal-med and abnormal-high respectively.

Table 11. Stratification of HPV-16 positive women by Pap and E6 RQ 0.5 test results with categories of cervical disease (n=121).

Pap result	Disease Category[a]					
	Normal		Abnormal Low		Abnormal Med & High	
	RQ+	RQ-	RQ+	RQ-	RQ+	RQ-
HSIL	0	1	1	1	43	11
LSIL	2	1	9	5	9	6
ASCUS	0	1	1	1	2	0
Normal	2	7	4	6	2	6
Total	4	10	15	13	56	23

[a] Largely derived from histological grading of biopsies obtained during colposcopic examinations with CIN1, CIN2 and CIN3 corresponding to abnormal-low, abnormal-med and abnormal-high respectively.

Table 12. Comparison of diagnostic test performance for the detection of CIN among women referred for colposcopy (n=887).

Histology / method	Sensitivity	Specificity	LR+[a]	LR-[b]
Any abnormality				
Repeat Pap[c]	0.68	0.75	2.74	0.43
HPV DNA	0.72	0.64	2.00	0.44
HPV Genotype	0.63	0.69	2.00	0.54
Pap or HPV DNA[d]	0.82	0.54	1.78	0.33
Pap or Genotype[d]	0.80	0.57	1.86	0.35
Pap and HPV DNA[e]	0.57	0.78	2.59	0.55
≥ CIN2				
Repeat Pap[c]	0.86	0.60	2.13	0.24
HPV DNA	0.89	0.53	1.91	0.20
HPV Genotype	0.84	0.62	2.18	0.26
Pap or HPV DNA[d]	0.96	0.40	1.61	0.10
Pap or Genotype[d]	0.96	0.44	1.73	0.09
Pap and HPV DNA[e]	0.79	0.72	2.82	0.29

[a] Positive likelihood ratio is given by LR+ = sensitivity / (1 – specificity)

[b] Negative likelihood ratio is given by LR- = (1 – sensitivity) / specificity.

[c] Includes ASCUS, LSIL or HSIL.

[d] Combined methods used the 'either positive' rule

[e] Combined methods used the 'both positive' rule

Predictive values provide the probability of disease given the test result and therefore are often used as a clinically relevant basis to compare test performances in a given population. However, predictive values are influenced by prevalence when test dependence between diseased and non-diseased groups is asymmetric (Gunnarsson & Lanke, 2002). Asymmetry can arise if, for example, there is a negative correlation between test results in the diseased population, but a positive correlation in the non-diseased population. Such is the case with HPV infection with its high prevalence of cytologically normal cervical smears in women that clear the virus without treatment. Consequently, likelihood ratios (LR) are useful for performance evaluation since they do not depend mathematically on disease prevalence and encompass the trade-offs between sensitivity and specificity. A combined test would have better test performance when $LR-_{combined} < LR-_{single}$ and $LR+_{combined} > LR+_{single}$. The single test is a better choice when $LR-_{combined} > LR-_{single}$ and $LR+_{combined} < LR+_{single}$ (Macaskill et al., 2002). If there is no simultaneous improvement in both likelihood ratios, then the choice between basing clinical decisions on a repeat Pap test and combining Pap with a molecular test is not obvious. In such a circumstance, there will be a trade-off between retaining true positives and eliminating false-positives. The assessment of trade-off using the Youden's index gives clinicians a straightforward basis for assessing whether there is potential value in using an adjunct test (Youden, 1950). The Youden's index can depict graphically with the trade-off per 100 persons tested (100T) as the prevalence of neoplasia varies

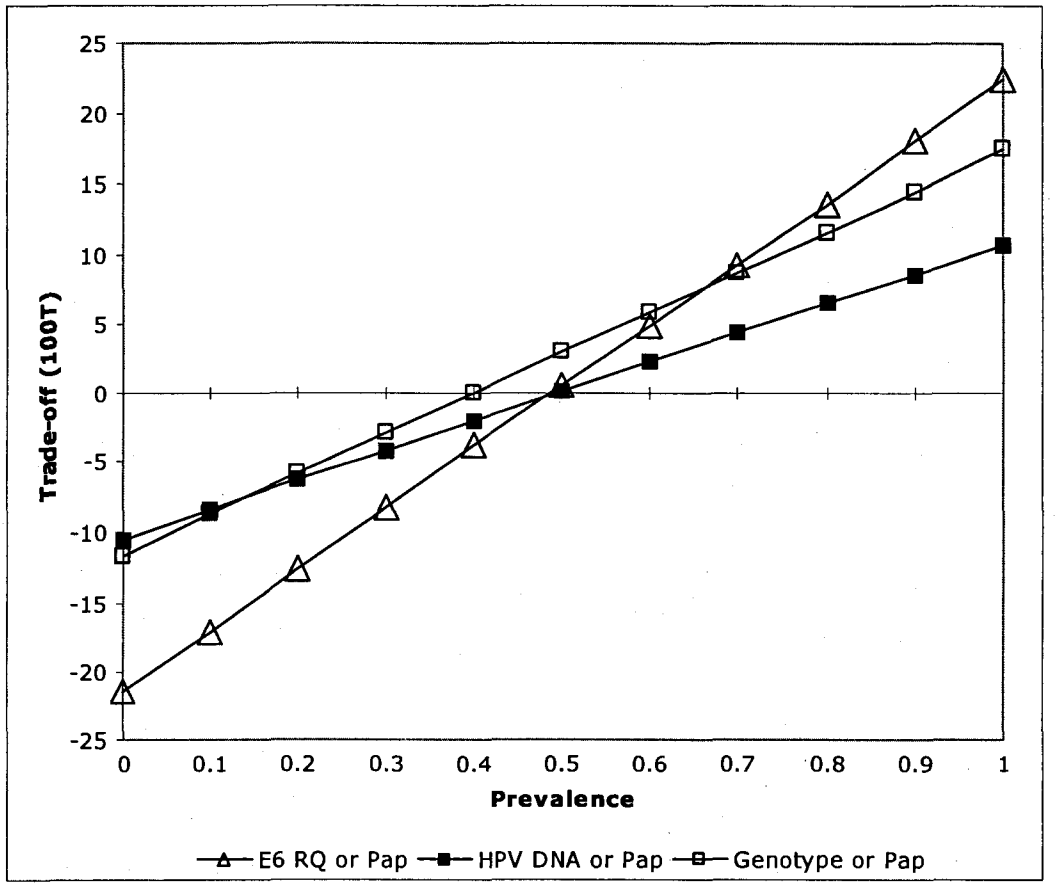


Figure 10. Trade-off in additional false positives per 100 persons tested. The point where each line crosses T = 0 indicates when the tests are equivalent for a predetermined acceptable ratio (R=1) of one false-positive for each true positive.

(Figure 10). It demonstrates the importance of considering prevalence when choosing the optimal single or combined test.

Given the LR information in Table 12, Pap testing offers an advantage combined with HPV DNA at higher disease prevalence for the detection of any grade of abnormality. However, genotype information offers an advantage combined with Pap over either as a stand alone single test regardless of prevalence for the detection of all grades of abnormality and retains that advantage at higher disease prevalence for the detection ≥ CIN2. Consequently, Pap and HPV genotyping, in combination, should be considered as an auxiliary testing tier before referral for biopsy, colposcopy or treatment. When using the combination for detection of ≥ CIN2, the auxiliary testing tier is best used in populations with disease prevalence > 0.4, which is likely after primary screening with abnormal Pap results as done here (Figure 10). The disease prevalence in our study population after two abnormal Pap results was 0.69. The actual effect of combination testing on the number of women excluded is indicated in Table 13. This additional combination of tests (Pap and HPV genotype - with 'either positive' rule) would have retained 96% of the women that needed further medical workup while deferring 267 (30.1%) from immediate colposcopy. Given that only 11% of women with CIN1 progress to CIN3 (Ostor, 1993), this auxiliary testing strategy would ultimately save up to 26.8% (n=238) of women in our triage population from colposcopy and potential problems associated with biopsy.

Table 13. Effect of cascade testing on triage numbers for the detection of severe CIN among women referred for colposcopy.

Cascade options	Normal-CIN1 (number excluded)	CIN2-CIN3 (number missed)
Tier 1		
Initial Screen (N=887)	603 (NA)	284 (NA)
Repeat Pap	242 (361)	243 (41)
HPV DNA	283 (320)	254 (30)
Pap or HPV DNA[a]	359 (244)	273 (11)
Pap or Genotype[a]	336 (267)	273 (11)
Pap and HPV[b]	166 (437)	224 (60)
Tier 2 (HPV-16 positive)		
Initial Screen (N=121)	42 (NA)	79 (NA)
Repeat Pap	23 (19)	71 (8)
HPV E6 RNA	22 (20)	68 (11)
E6 RQ	19 (23)	56 (23)
Pap or HPV E6 RNA[a]	31 (11)	73 (6)
Pap or E6 RQ[a]	29 (13)	73 (6)

[a] Combined methods used the 'either positive' rule

[b] Combined methods used the 'both positive' rule

3.2.4 Utility of HPV RNA as an adjunct test

Taking the cascade testing to the next level (e.g., Tier 2), the potential utility of HPV E6 RNA or the more refined analysis of E6 RQ was examined. That is, could knowledge of the level of HPV transcripts be useful as a complement test for triage. A sample was considered positive by E6 RQ if it had a value of 0.5 or greater (refer to 3.3.8). Again, the 'either positive' rule was chosen to improve test performance in the diseased group and was compared with repeat cytology. Table 9 and 10 shows the test and reference standard results on 121 HPV-16 positive study samples for HPV E6 RNA detection and E6 RQ respectively. The prevalence of disease in patients with HPV-16 with any type of CIN was 0.88 and 0.65 for CIN2 or greater. In this subset of women, there was still 34.7% with ≤ CIN1 (Table 9). Additional testing of clinical material already collected could be a useful reflex testing strategy to further eliminate unnecessary biopsy and colposcopy.

The test performance measurements of HPV relative quantification analysis are summarized in Table 14. Again no single test demonstrated the best combination of sensitivity and specificity. The simple detection of HPV E6 RNA (i.e. positive/negative scoring for E6 transcript presence) offered no advantage as a single auxillary test or in combination with Pap testing. However, the relative quantification of the E6 transcript (i.e. E6 RQ) did offer some further advantage for triage, when standardized to HPV viral load, the number of cells collected and the yield of the nucleic extraction as done here for the first time.

Table 14. Comparison of diagnostic test performance for the detection of CIN among HPV-16 positive women (n=121).

Histology / method[a]	Sensitivity	Specificity	LR+[b]	LR-[c]
Any abnormality				
Repeat Pap[d]	0.83	0.64	2.33	0.26
HPV E6 RNA	0.79	0.57	1.83	0.38
E6 RQ	0.66	0.71	2.32	0.47
Pap or HPV E6 RNA	0.89	0.36	1.38	0.31
Pap or E6 RQ	0.89	0.50	1.78	0.22
≥ CIN2				
Repeat Pap[d]	0.90	0.45	1.64	0.22
HPV E6 RNA	0.86	0.48	1.64	0.29
E6 RQ	0.71	0.55	1.57	0.53
Pap or HPV E6 RNA	0.92	0.26	1.25	0.29
Pap or E6 RQ	0.92	0.31	1.34	0.25

[a] Combined methods used the 'either positive' rule

[b] Positive likelihood ratio is given by LR+ = sensitivity / (1 – specificity)

[c] Negative likelihood ratio is given by LR- = (1 – sensitivity) / specificity.

[d] Includes ASCUS, LSIL or HSIL.

Examination of the LR for the combination of Pap testing with E6 RQ showed that for higher disease prevalence the combined test would find more true positives than simply an additional Pap test (Figure 10). In other words, the addition of E6 RQ could be used to further identify 31.0% (n=13) of HPV-16 positive women with ≤ CIN1 while retaining 92.4% of women with CIN2 or greater for triage to colposcopy (Table 13).

3.3 Protocol development and validation

The study has a strong biological rationale. The targeted biomarkers were expected to be differentially expressed in normal versus high-risk tissue. It was expected that the marker would appear early in the staging towards carcinogenesis since it was hypothesized to measure the cause of disease and not just a surrogate marker of it. One issue was how straightforward the marker would be to measure. It is this aspect of the research in which unprecedented gains in the field have been made with RNA technologies coupled to real-time RT-PCR.

This study also had the following advantages: 1) 'strength-of-design' in that colposcopy patients were a good study population including retrospective and prospective portions with the latter completely blind until the conclusion of the research, 2) best possible technical methodology (i.e., top-of-the-line reagents and equipment), providing reproducibility, 3) triplicates for all quantification assessements 4) very stringent quality control measures and 5) validation of methods and procedures before, during and after the study to

ensure quality data. When appropriate, the study exercised practicality without sacrificing the quality of the results, e.g., there was the policy to repeat PCR negatives with a second target and the follow-up any indeterminate genotypes with a third method.

3.3.1 Validation of cell processing and RNA preparation protocol

Immediately after collection, cervical brushings were stored in ethanol and transported on dry ice to the DNA Lab where the RNA in the samples was stabilized with TRIzol and kept at -70°C. This protocol for cervical cell processing and RNA extraction was based on previously published findings regarding RNA stability and extraction (Bachoon et al., 2001; Florell et al., 2001; Lamarcq et al., 2002; Soukup et al., 2003; Van Deerlin et al., 2002). It had already been shown that cervical cells scraped from the cervix exhibit a high proportion of degraded RNA despite immediate freezing in liquid nitrogen, regardless if they were put immediately in TRIzol, cell culture medium, or ethanol (Lamarcq et al., 2002). However, the effect of time-to-processing had not been studied, which was a variable in this study due to the physical distance between the hospital and the laboratory and the time delay between the sample being taken and at hand for processing. Consequently, total RNA within the collected cells could not be stabilized with the TRIzol reagent for up to 8 h. Since RNA is susceptible to degradation, the maximum time-delay between collection and nucleic acid stabilization needed to be established. Therefore, the collection protocol was tested with cell-line material before it was applied to clinical specimens to determine if any measurable affect could be attributed to the delay

in processing, or if ethanol and freezing would mitigate the time-delay between collection and TRIzol stabilization. After homogenization in TRIzol and before addition of chloroform, the manufacturer stated that samples can be stored at -70°C for at least one month before any measurable degradation occurs. This RNA stabilization effect of TRIzol has been demonstrated with Western Equine Encephalitis virus and West Nile virus in mosquito homogenates (data not shown).

SiHa cells were resuspended in ethanol to a concentration of $1x10^5$ cells/ml. Twenty-four 1 ml aliquots of the prepared SiHa cell suspension were then placed on dry ice until six replicates were removed from the dry ice for processing at the following time (h) intervals from the freezing event: 1, 24, 48, and 72. At the appropriate time, the cells were processed and then stored in TRIzol at -70°C until the other aliquots were processed with the last set of aliquots frozen with the rest for 16 h before extraction and testing of all aliquots were performed simultaneously. Figure 11 shows the amount of transcript as measured by real-time RT-PCR. Transcripts were found to be stable for 48 h when kept in ethanol and on dry ice, which was within the time frame for specimen processing in this study.

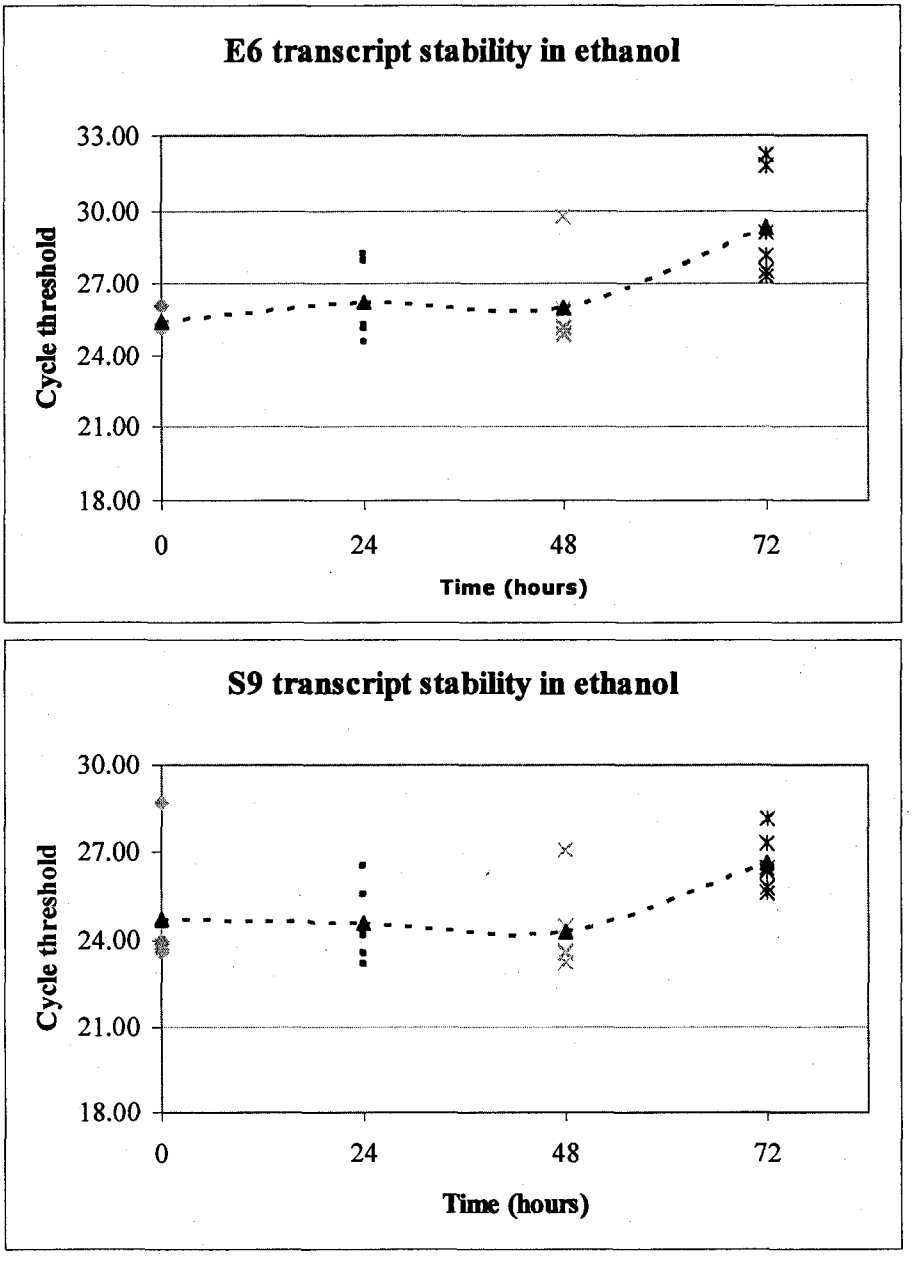


Figure 11. Transcript stability in ethanol.

Aliquots of cell suspensions were processed at four time intervals to ensure nucleic acid stability. An increase in cycle threshold of 3.3 cycles indicates a log reduction in amplifiable template. The upper graph shows the levels of HPV-16 E6 transcripts over time, whereas the lower graph shows the levels of S9 Human transcript levels. All six replicates are plotted at each time point while the average Ct value is plotted with the trend line.

3.3.2 Validation of HPV screening and genotyping

Samples were separately analyzed at the National Microbiology Laboratory (NML) to permit a comparison with the local HPV detection and genotyping results. An aliquot of extracted DNA from seven archived specimens was separately analyzed before the beginning of the prospective study period. There was 100% congruence with HPV detection in that both laboratories found the same five samples to be HPV positive. There was agreement with four of the five HPV genotyping results. The one discrepant sample was identified to be HPV-74 by the DNA lab, but HPV-42 by the NML. The E1 PCR product from the discrepant sample was run on a longer gel, thereby resolving two bands, which were then excised and sequenced separately. This confirmed that the discrepant sample was a mixed infection (with HPV-42 and HPV-74) and it was concluded that each protocol was preferentially amplifying the other genotype. The identification of mixed infections through DNA sequencing is known to be problematic and has been found to miss 40% of mixed HCV infections (Hu et al., 2000).

Approximately halfway through the study period, an extra pellet of cells was prepared for study numbers 355 through 435. These 79 consecutive clinical specimens were split into four aliquots with three of the aliquots processed as described above. The fourth aliquot was kept at -70°C until it was shipped on dry ice to the NML for HPV detection and genotyping using nested L1 PCR and DNA sequencing. All 35 HPV positive samples determined by the DNA lab were repeat positives by the NML, indicating no false-positive results. However, the NML found an additional 11 samples to be positive using a more sensitive nested

PCR. These positives were determined to be HPV-51 (n=3), HPV-52 (n=2), HPV-31 (n=2), HPV-16 (n=1), HPV-53 (n=1), HPV-56 (n=1) and HPV-62 (n=1). Consequently, the concordance for HPV detection between the two laboratories was 86% (68/79).

For the remaining 46 concordant HPV positive samples, there was 85% (39/46) agreement on the HPV genotype between the two laboratories. Missed mixed genotype determinations accounted for 2/7 differences, whereby the DNA lab found HPV-16/31 and HPV-31/59 whereas NML found HPV-16 and HPV-31 respectively. The other five differences pertained to completely different HPV genotype determinations between the two laboratories. Samples 357, 360, 374, 386 and 435 were called HPV-74, HPV-67, HPV-86, HPV-39 and HPV-45 by the DNA lab whereas the NML identified these respective samples as HPV-58, HPV-73, HPV-66, HPV-87 and HPV-56. It is inferred that these discordant results were further examples of preferential detection of one genotype among a mixed infection due to protocol bias.

In one example, the genotype difference changed the risk classification from high-risk to unclassified. For specimen 386, the genotype identified by the DNA lab (HPV-39) is classified as high-risk, whereas the genotype identified by NML (HPV-87) is currently not classified. The diagnosis for this patient was normal based on the Pap result with no histology done at the time of study sample collection. However, a biopsy taken 12 months earlier for this patient indicated CIN1, but no HPV testing was done during that visit.

It has been shown that the Gp5+ & Gp6+ primers (i.e., L1 PCR) could miss HPV-52 (Chan et al., 2006b). In this study, all nine HPV-52 genotype determinations were made with E1 PCR and not with the L1 PCR, thus supporting the previous finding.

For indeterminate genotypes within the study, qualitative judgment of PCR product quantity was made by visually inspecting the gel photos to determine which of the samples had weak PCR products. It appeared that, for 9/41 prospective indeterminate samples, the amount of PCR product generated was not the limiting factor but rather it was the sequence quality, as indicated with peak-under-peak noise. Noise in sequence can be caused by a mixed template and it was expected that multiple infection was a common cause for these indeterminate genotypes.

The other 32 indeterminate genotypes were from samples that produced a very weak PCR product. Consequently, after purification of the PCR product, the quantification gel indicated that these samples were well below the recommended input for a successful DNA sequencing reaction. Nevertheless, these samples were used undiluted for sequencing. In fact, for a few of these samples, the PCR product was not visible on the quantification gel and only poorly visible on an agarose gel before purification. Six of these 32 samples did not yield enough sequencing signal for any nucleotide sequence determination, whereas, the others did yield readable sequence but had very poor quality or short reads and therefore did not give a match to a GenBank HPV sequence

using the analysis criteria (score above 90% match with ≥100 nucleotides queried).

Two of the indeterminate samples were run with the β-actin real-time PCR and this assay indicated that a reasonable amount of human DNA was present in the preparation. This result was expected since all specimens had plainly visible cell pellets. Furthermore, evaluation of the DNA extraction protocol (data not shown) produced results that would indicate consistent and good DNA recovery and purity. Therefore, problems with quantity or PCR inhibition were not indicated as contributing factors with the amount of indeterminate genotypes. Rather, the low HPV PCR products were likely due to a combination of low viral load within infected cells and/or low numbers of infected cells collected within a background of uninfected cells. At least 23/41 of these indeterminate samples were non-HPV16 or non-HPV31 since the genotype-specific real-time PCR assays for these two HPV-types were also negative.

Luminex® technology at the NML was used on the 41 samples to help resolve the genotype. This technology found 8/41 samples to be mixed. It is impossible to determine from the data collected here, or any other study to date, if more than one HPV genotype is capable of infecting the same cell or if mixed infections are a result of two populations of cells, one infected with one genotype and the other infected with a different one.

3.3.3 Validation of real-time PCR optimization

The effect of efficiency on accurate real-time quantitative PCR is exponentially dependent on cycle number (Liu & Saint, 2002). For accurate

relative quantification, the efficiency of the two PCRs must be the same. Accordingly, the slope of an external standard curve for each real-time assay was measured over the dynamic range within which the clinical samples were expected. Effort was paid during optimization to get the efficiency of each PCR to be as close to the ideal as possible so that the expected error due to effect of efficiency differences between standards and unknowns was minimized. The efficiency of PCR was measured over a range of initial template concentrations and then analyzed by plotting the Ct against the log of the initial template concentration (data not shown). The slope of the line was used to determine the PCR efficiency (E) whereby $E = 10^{(-1/slope)}$. The estimated efficiencies were as follows: 2.06 for the 16E6 DNA PCR, 1.98 for the 16E6 RT-PCR, 2.03 for the 16E7 PCR, 1.99 for the 16E7 RT-PCR, 1.91 for the 16L1 RT-PCR, 1.91 for the β-actin PCR, and 2.05 for the S9 RT-PCR. The reproducibility was excellent as evidenced by the fit of three replicates at each concentration to the trend-line using the correlation coefficient (R^2). R^2 was ≥ 0.998 for all plots.

3.3.4 Validation of prospective sample size

There were 785 samples collected during the prospective study period of which 131 were HPV-16, of these 128 passed the RNA quality assessment, 121 passed run quality control, which were collected from 108 different patients. Fifty-six samples were determined to be CIN3, whereas the remainder of the samples were categorized as one of the following: CIN2 (n=22), CIN1 (n=28) or normal biopsy (n=15).

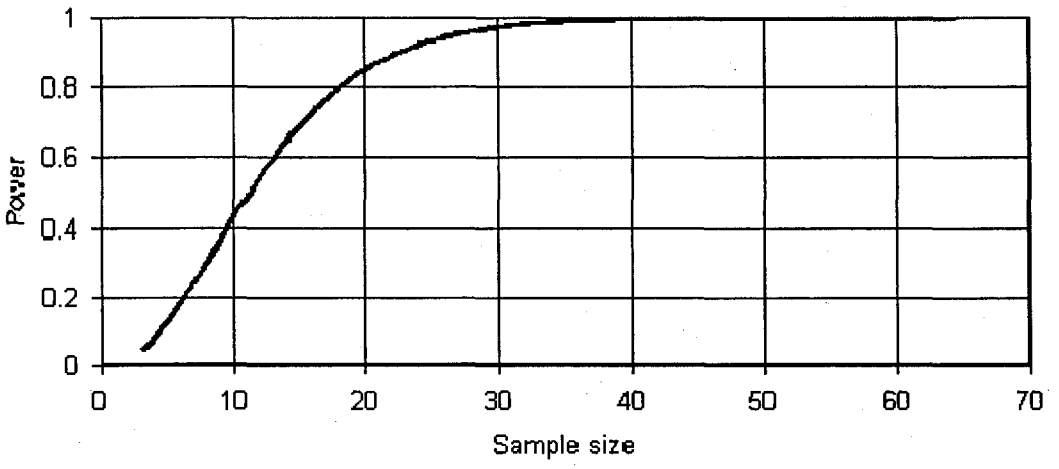


Figure 12. Power for dichotomous outcome of CIN3 with the E6 RQ test. The plot indicates the power of the test with the following parameters; Type-I error probability for a two-sided test set to 0.05, 56 case patients, 65 controls (prospective), probability of event rate among controls 0.46, and probability of event rate among cases 0.80.

The power of relative quantification by real-time RT-PCR for HPV-16 E6 RNA (henceforth called E6 RQ), using the ROC-determined cutoff of 0.5, was calculated for the correct dichotomous identification of CIN3 (Figure 12). The calculation shows that the final sample size of 121 HPV-16 positive samples had the power of 0.9999 for detecting a specific alternative hypothesis with a specified Type-I error probability. In other words, given the sample size and test performance specifics calculated with the Fisher's exact test, >99% of repeat studies with the same sample size will achieve statistical significance (P value of ≤0.05). This means that all repeat analysis using E6 RQ will produce results that support the findings here. This indicated that the sample size used here was adequate to support the finding that E6 transcript levels are, on average, higher in cases of CIN3 compared to all other histological outcomes.

3.3.5 Validation of relative quantification

Real-time PCR was used for nucleic acid quantification because of its demonstrated and unsurpassed sensitivity and high dynamic range (Gravitt et al., 2003; Martell et al., 1999). Essentially, the 5'-3' exonuclease activity of *Taq* DNA polymerase was utilized to detect PCR products via the generation of a fluorescent signal after target-specific PCR-dependent probe degradation by the polymerase enzyme (Heid et al., 1996). Förster Resonance Energy Transfer (V. T. Förster. Ann. Phys. 2:55-75, 1948) prevented the fluorescence signal via the coupling of a fluorogenic dye molecule (reporter dye) and a quencher moiety (quencher dye) to the same target-specific probe. The probes were exonuclease oligonucleotide substrates with a 5' reporter dye (6-carboxyfluorescein), a 3'

quencher dye (Black-Hole quencher), and a phosphate-blocked 3' end. The sequence of the oligonucleotide was based on the complementary sequence of the target gene for which it was designed to anneal. The physical proximity of the reporter dye and the quencher dye resulted in the suppression of reporter fluorescence. However, when the probe is annealed 3' of either primer during PCR, then the 5'-3' exonucleolytic activity of *Taq* DNA polymerase degrades it, allowing the reporter dye to diffuse away from the quencher dye resulting in an increase in reporter fluorescence.

The increase in fluorescence, which has been shown to be transiently related to the amount of target nucleic acid, was measured during the extension phase of the PCR using an ABI 7700 (Applied Biosystems). The Ct was determined for each reaction and it represents the cycle number at which significantly increased fluorescence was first detected. The threshold is a numerical value that was assigned for each run and was determined manually after baseline correction was applied automatically by the software for the ABI 7700 instrument. The threshold was typically set to 0.2 relative fluorescent units but was determined for each run based on the amplification plots such that Ct values were at the beginning of the linear portion of the logarithmically plotted fluorescent curve vs cycle number. Determination of Ct in this way ensured that the fluorescence most accurately reflected the amount of initial target nucleic acid. Ct is considered the most accurate measurement for quantification since the accumulation of PCR products is the most exponential at Ct, whereas later, the PCR efficiency is known to plateau as product accumulates (Jung et al.,

2000). In other words, PCR amplification is template concentration dependent but reactions with low starting copy number can reach the same plateau as reactions that started with a higher template concentrations and/or different PCR efficiency. However, real-time measurements and Ct determination prevents errors introduced by the plateau effect.

The efficiency of the reverse transcriptase activity of *Taq* DNA polymerase has been shown to be ≤1% compared to avian myeloblastosis virus (AMV) reverse transcriptase (Jones, 1993). This inefficiency of *Taq* DNA polymerase for the conversion of RNA to cDNA as the necessary substrate for PCR was utilized as substantiation of DNA-free RNA preparations. In other words, no increase in fluorescent signal in PCR but target amplification in RT-PCR with the same primers and probe signified the presence of amplifiable RNA target that was free from contaminating DNA. It should be noted that reaction efficiencies of the PCR and RT-PCR with the same oligonucleotides were demonstrated to be similar (see 3.3.3).

The most appropriate statistical test to examine the significance of relative quantification analysis was a nonparametric test (i.e., Kruskal-Wallis) because some values were "off the scale". That is, the values were too low to measure because the RNA levels were below the sensitivity limits of the real-time assay or simply not there. Nevertheless, a normality test was performed for each data set. The D'Agostino-Pearson normality test (omnibus K2) first computes the skewness (how asymmetrical is the distribution) and the kurtosis (how far away from a Gaussian shape). It then calculates how far each of these values differs

from the value expected with a Gaussian distribution, and computes a single P value from the sum of these discrepancies.

Normality testing showed that the relative quantifications of the RNA transcripts produced data that were non-Gaussian. Inspection of kurtosis and skewness values (data not shown) supported that the data were not normally distributed. Determination of the best relative quantification approach was conducted using the Kruskal-Wallis statistical test with the same data set as the final analysis. The following preliminary analyses were conducted before final analyses were made for comment on correlation with patient diagnosis and Pap results.

3.3.6 Repeat sample analysis

Seven specimens were not successfully linked to the patient information. As a result, 1362/1369 study specimens were linked with 1166 patients. Some specimens originated from the same patient from more than one visit to the clinic during the study period. As a result, 156 patients had two specimens, 16 patients had three specimens and two patients had four study specimens taken during the course of the study. Seventy percent (121/174) of these repeat patients had no change in the HPV status (i.e., PCR result or HPV genotype) whereas 17% (30/174) demonstrated a change in HPV positivity (i.e., PCR positive to PCR negative) while 13% (23/174) of the patients demonstrated a change in HPV genotype between visits. In fact, two patients actually exhibited a different genotype at each of three visits. Consequently, this works supports a

previous finding that women can be reinfected with a different HPV genotype (Richardson et al., 2003).

For the patients with repeat samples, there were 112/174 that provided two or more samples and also had defined Pap results (i.e. excluding patients with any ASCUS or missing Pap data). These repeat patients were analyzed for congruence between repeat visits by comparing the pair of results (i.e., Pap result and HPV status) at each visit. There were eight-four (75%) visits that demonstrated agreement between the paired Pap result and HPV status. That is, either the Pap and HPV results were identical from one visit to the next, or if there was a change in Pap result, then there was a corresponding change in the HPV status. Moreover, it was found that a change in a patient's Pap status was correlated with a change in the patient's HPV status 76% of the time (34/45). Likewise, no change in Pap status was correlated with no change in HPV status 75% of the time (50/67). These repeat testing results confirmed that the correlation of Pap and HPV status was significant
($P < 0.0001$).

It could be argued that any difference between the Pap test and HPV status with follow-up samples on the patient was due to errors in testing. Indeed, 12/17 discordant repeats that were the same by the Pap test, yet with a different HPV status, were substantiated by histology (i.e., histology supported the Pap test result). However, six of the twelve were different in HPV status because of a change in HPV genotype. Consequently, a false-negative PCR could not be ruled out for 35% (6/17) of the discordant cases. Not all occurrences where a

different Pap test correlated with a different HPV status were due to viral clearance and subsequent reversion to normal cytology. In fact, only 68% (23/34) of the cases appeared consistent with such a scenario. Interestingly, 23% (7/30) of the abnormal to normal Pap test result changes were accompanied by an HPV genotype change. It is possible that these cases would be typified by clearance of a high-risk genotype, thereby possibly revealing the low-risk type in a mixed infection. In fact, 4/7 cases were from one high-risk type to another high-risk type whereas 2/7 involved a high-risk and an unclassified-risk genotype. Interestingly, one of the cases involved a switch from a low-risk type (HPV-6) to a high-risk type (HPV-39). Consequently, reversion to a normal Pap test without complete HPV clearance is possible. This supports findings that HPV detection alone may not be the most reliable indicator to estimate whether cervical abnormalities will progress or resolve (Monnier-Benoit et al., 2006).

Nevertheless, a positive HPV DNA result increases the likelihood of an abnormal diagnosis by almost twofold (1.96). However, the sensitivity of HPV DNA for the detection of an abnormal cytology is only 0.71 with a poor NPV of only 0.49. On the other hand, the sensitivity of the HPV DNA testing algorithm used here for the detection of HSIL was 0.90 and is better than most published sensitivities, which range from 0.68 to 0.98 and average 0.85 (Ghim et al., 2002).

The samples for Pap and HPV DNA detection and genotyping in this study were taken before the biopsy. Consequently, the performance of these three tests (Table 2) could be considered as they might perform when the patient visits a specialist's office and before the scheduled colposcopy. The NPV (0.91) of

HPV DNA testing for ≥ CIN2 could be used to decide if triage to colposcopy is warranted. Further, if the patient is HPV DNA positive then the NPV of HPV genotyping with risk classification could be used to avoid unnecessary colposcopy of women that have a low-risk HPV type. The identification of a low-risk genotype precludes, with reasonable certainty, the diagnosis of ≥ CIN2 (NPV ≥ 0.83). However, the possibility of mixed HPV infection or reinfection needs to be considered. The follow-up of treated patients using HPV DNA testing needs to be interpreted differently from test results before treatment since an HPV DNA positive result does not necessarily mean treatment failure. The detection of HPV DNA 2 to 3 months after treatment was consistent with a study that found clearance of an HPV infection after treatment only after 3-6 months (Costa et al., 2003).

3.3.7 Best relative quantification analysis

The results from the relative quantification of the HPV-16 E6 viral transcripts were examined on the ability to correctly categorize patients/samples as either healthy (i.e., normal histology) or with one of three levels of cervical neoplasia (i.e., abnormal-low, abnormal-medium or abnormal-high). Essentially, there was one raw data set, which was generated using the real-time PCR and real-time RT-PCR (refer to 2.12). However, the quantity of HPV-16 E6 RNA was calculated with one of seven treatments/methods (see methods section 2.15). Each treatment was a different approach for correction of variables that were thought to increase variability of results. Ideally, for a diagnostic test, there should be significantly different medians between diseased and non-diseased

individuals with no overlap among the range of results for each category. Upon inspection of the data this was clearly not the case and so the diagnostic value or superiority of any treatment is not immediately obvious (Table 15).

The results from the different analytical approaches are summarized in Table 16. The Table lists the seven alternative treatments of the relative quantification data (treatment detailed in 2.15) and the resulting P and KS values from separate Kruskal-Wallis analyses. The KS value was used to indicate how each treatment performed in the correct assignment of women as either having a 'normal' cervix or one habouring cells that could be precursors to cancer. Each successive method therotically generated more correction for variation over the previous method. The introduction of standard error with 'assay correction' was observed since the treatments get worse (i.e., lower KS value) after method 4. Method 4 generates the greatest difference in medians between groups. Therefore, the relative quantification of HPV transcripts with standardization to total RNA, total DNA and amount of HPV DNA was determined to have the best correlation to diagnosis. In other words, the treatments before 'standardization to total RNA/DNA & HPV DNA' have experimental error (i.e. methods 1-3), while the treatments attempting to correct for interassay variablity (i.e. methods 5-7) suffer from more introduced standard error than any applied correction for experimental error. Consequently, all analysis of RQ of HPV transcripts used method 4 for the calculation, which is a novel approach for RQ.

Table 15. Mean level of HPV-16 E6 transcript by category of disease among HPV-16 positive women referred for colposcopy.

	Mean (95% CI)			
	High	Medium	Low	Normal
Method 1	2.3 (2.00 ~ 2.60)	1.5 (0.98 ~ 2.03)	1.5 (0.94 ~ 2.08)	0.9 (0.24 ~ 1.57)
Method 2	0.6 (0.54 ~ 0.68)	0.4 (0.31 ~ 0.57)	0.4 (0.26 ~ 0.55)	0.2 (0.05 ~ 0.39)
Method 3	0.8 (0.59 ~ 1.16)	0.3 (0.23 ~ 0.46)	0.4 (0.23 ~ 0.51)	0.2 (0.05 ~ 0.35)
Method 4	1.1 (0.76 ~ 1.53)	0.4 (0.3 ~ 0.58)	0.4 (0.28 ~ 0.61)	0.2 (0.06 ~ 0.46)
Method 5	2.6 (1.62 ~ 3.49)	0.9 (0.64 ~ 1.15)	0.9 (0.58 ~ 1.30)	0.5 (0.13 ~ 0.84)
Method 6	7.0E+11 (-4.51E+11 ~ 1.85E+12)	1.96E+13 (-1.15E+13 ~ 5.07E+13)	2.99E+14 (-2.29E+14 ~ 8.27E+14)	1.60E+13 (-1.12E+13 ~ 4.31E+13)
Method 7	1.24E+10 (-7.28E+09 ~ 3.21E+10)	7.36E+10 (-3.12E+10 ~ 1.78E+11)	1.06E+12 (-4.06E+11 ~ 2.52E+12)	9.46E+10 (-9.10E+10 ~ 2.80E+10)

Table 16. Best formula for RQ analysis of HPV transcripts

Method[a]	P value	KS[b]
1) Raw concentration	0.0007	17.07
2) Standardized to Human RNA	0.0007	17.14
3) Standardized to HPV DNA	0.0003	18.68
4) Standardized to total RNA/DNA & HPV DNA	<0.0001	22.77
5) Method 4 and correction for run-to-run variation	<0.0001	21.37
6) Method 5 plus correction for PCR efficiency	0.0148	10.49
7) Method 5 plus individual PCR efficiency correction	0.0060	12.43

[a] Method 1 is not relative to any other gene, Methods 2-4 are target concentrations divided by reference concentrations. Methods 1-5 calculate concentrations using an external standard curve (equation 2). Methods 6 and 7 use equation 1. Details of the calculations can be found in section 2.15.

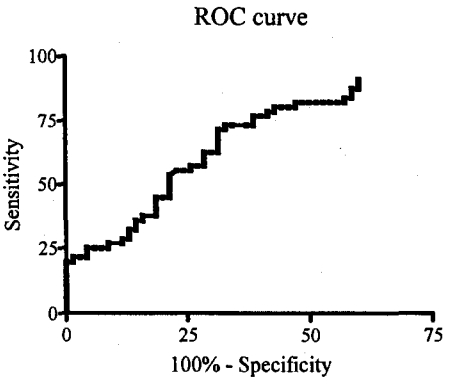
[b] Kruskal-Wallis statistic

A

High vs Medium-Normal

Area	0.7307
P	<0.0001

Cutoff	**Sens**	**Spec**
>0.0983	91.1%	40.0%
>1.123	19.6%	100.0%

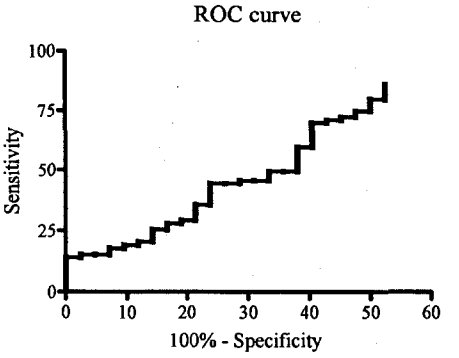


B

High-Medium vs Low-Normal

Area	0.6631
P	0.003238

Cutoff	**Sens**	**Spec**
>0.0983	86.1%	47.6%
>1.123	13.9%	100.0%



C

High-Low vs Normal

Area	0.719
P	0.007889

Cutoff	**Sens**	**Spec**
>0.0983	78.5%	57.1%
>1.020	15.9%	100.0%

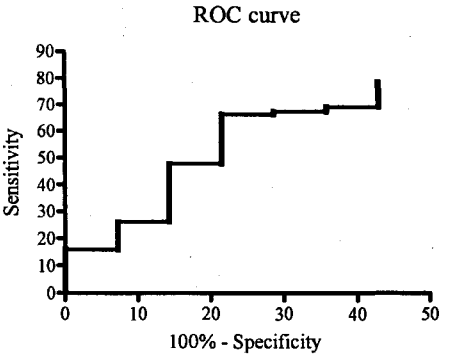


Figure 13. ROC curves for HPV-16 E6 RNA RQ.

ROC curves depict the relation between sensitivity and specificity over the range of possible cutoff values based on HPV-16 E6 RNA RQ. The ROCs for the three possible dichomizations of patients based on clinical diagnosis of the cervix are shown in panels. Adjacent to each ROC are the two cutoffs of HPV-16 E6 RNA RQ that achieve the highest possible sensitivity (sens) and specificity (spec). The area under the curve and P values are also included for each ROC.

Intuitively, method 4 would provide the best 'biological' standardization since it attempts to account for the amount of specimen collected and analyzed while correcting for any degradation and losses in yield during extraction. At the same time, it attempts to quantify the amount of transcript per viral genome copy and therefore accounts for transcript level differences due to viral load.

3.3.8 Best cutoff for dichotomization of RQ data

Receiver operator characteristic (ROC) curves (Nettleman, 1988) for HPV-16 E6 RQ are shown in Figure13. A cutoff of ≥0.5 was chosen as a positive result for HPV-16 E6 RNA RQ, which gave the best trade-off between sensitivity (0.66) and specificity (0.78). Alternate cutoffs of 0.9 and 1.0 were examined in attempt to enhance the determination of abnormal-high diagnosis. These cutoffs resulted in specificities of 0.90 and 0.95 respectively. However, the sensitivities dropped to 0.27 and 0.25 respectively. The calculation of sensitivities and specificities other than for the dichomization of abnormal-high and all others were not possible since the Fisher test showed that these higher cutoffs could not be evaluated with statistical significance. As can be expected, raising the cutoff to increase specificity reduced the number of samples retained above the cutoff. There were 75 samples with values above a cutoff of 0.5, while only 22 and 18 samples had cutoffs of 0.9 and 1.0 respectively.

3.4 Conclusions

The use of HPV E6 RNA as biomarker for cervical cancer has a strong biological rationale. The targeted biomarkers E6 and E7 are known to be

required for the transformed phenotype in continuous cell-lines derived from HPV-infected tissue (Demers et al., 1994; Nauenburg et al., 2001). However, the examination of the use of HPV oncogene transcripts in a diagnostic setting invariably leads to the same conclusion. Namely that, there is a correlation of higher levels of the oncogene transcripts with cervical neoplasia, but the predictive value remains low (Czegledy et al., 1994; Daniel et al., 1995; Hsu et al., 1993; Ke et al., 1999; Park et al., 1997; Selinka et al., 1998; Sotlar et al., 1998; Sotlar et al., 2004; Wang-Johanning et al., 2002). HPV RNA shows great potential and has been the focus of a new molecular test, the PreTect HPV Proofer (NorChip AS). It is reasonable to expect that HPV E6 RNAs would make good markers since they appear early in the staging towards carcinogenesis. These transcripts and their presence or even their relative levels correlate with the 'cause' of disease and so therefore, they should be more than a surrogate marker for cervical cancer.

However, the unresolved issue is how easily the marker can be measured in clinical material. It is this aspect of the research in which unprecedented gains in the field have been made relatively recently with the advent and sophistication of real-time RT-PCR and other biotechnologies surrounding RNA work (Mackay et al., 2002; Stahlberg et al., 2005). This study attempted to capitalize on the most recent advances in chemistry, instrumentation and the latest in mathematical modeling. Sufficient clinical material was obtained and final clinical diagnosis were used to ensure the most accurate test evaluation possible.

Consequently, all effort has been made towards the elimination of variables masking any true diagnostic potential of HPV E6 RNA.

This work demonstrated, for first time, that there is a diagnostic utility in the analysis of HPV transcription and that it benefits from standardization for variables such as amount of HPV DNA and total cellular nucleic acid. The analysis of E6 transcript levels combined with Pap testing has a demonstrated diagnostic utility in a triage protocol. The added gain in diagnostic performance is attributable to the high specificity of E6 RQ analysis. Future improvement to E6 RQ analysis could be made if the actual number of infected versus uninfected cells could be included in the standardization, but this is currently not technically possible. In any case, this added standardization, when possible, would need to be evaluated against and with the novel approach of standardization to HPV DNA presented here.

Interestingly, E6 transcript levels can be used to increase the specificity over simply presence/absence scoring of the transcript. An application of the E6 RQ could be used to confirm an abnormal diagnosis (positive predictive value 95%). In this circumstance, the likelihood ratio supports relative quantification of E6 transcript to be better than simply E6 RNA +/- for the detection of cervical neoplasia of any grade. This study supports earlier findings that E6 and E7 transcription levels are typically elevated as patients' progress toward cervical cancer. Furthermore, this study found for the first time that L1 transcript levels did not seem to correlate with neoplastic progression.

The utility of E6 RQ as triage tool was shown by the study but its potential for prognosis is perhaps the most intriguing. Further study with patient follow-up may find that RNA molecular tests are better for prognosis than diagnosis. In this study, 3/3 patients that were below the 0.5 cut-off for RQ of E6 and with a biopsies of CIN1 remained CIN1 after 4-5 months when another biopsy was taken. On the other hand, 4/8 CIN1 patients that were positive for E6 RQ 0.5 progressed to ≥ CIN2 in 3-10 months. Nevertheless, prognostic value evaluation will need a retrospective cohort study.

The use of HPV genotyping in combination with a Pap screening program demonstrates excellent diagnostic potential. HPV DNA testing has already been demonstrated to be useful for reflex testing of Pap smears with atypical squamous cells of undetermined significance (Srodon et al., 2006). The addition of HPV genotyping regardless of the Pap smear result to any triage protocol was shown here. The study showed that HPV genotyping would eliminate several unnecessary procedures for women and consequently focus resources of cervical cancer prevention programs.

There were a number of statistically substantiated findings including the observation that older women who are referred to colposcopy (age ≥30) tend to have a better chance of a normal diagnosis of the cervix than younger women (age<30). Pap testing was found to be more specific than PCR testing (>90%) but not very sensitive (<60%). This high specificity of Pap testing highlights the superior performance of the cytology program within the health region. A systematic review of the accuracy of the Pap test has calculated, for thresholds

CIN2 to CIN3, that the average Pap sensitivity is 58% with the specificity at 92% (Nanda et al., 2000). Qualitative HPV DNA testing (i.e. +/- PCR score) is more sensitive than Pap testing (>70%). However, HPV DNA testing is not very specific (<64%) compared to Pap. However, this is not unexpected as the specificity for HPV DNA is similar to other studies. For example, 64.1% was the specificity of HPV DNA testing for identifying women with cervical neoplasia among equivocal Papanicolaou results from a cohort of 46,009 women who had routine cervical examinations (Manos et al., 1999). HPV genotype risk classification is very sensitive (>97%) but has very poor specificity for cervical disease (<14%).

Perhaps the most interesting observation emerging from the correlation studies is that HPV-31 appears to have the same high/medium risk as HPV-16 for cervical disease and is more "high-risk" than HPV-18 in the Saskatchewan population. The evaluated risk of HPV-31 could be the cause for its increased prevalence in women seen at the colposcopy clinic. This knowledge of base-line HPV prevalence will be invaluable for measuring the success or failure of the HPV vaccine in our population. The vaccines recently licensed for use are directed against high-risk HPV types 16 and 18 and the low-risk types 6 and 11. Consequently, there are over 20 high-risk HPV types that are not currently covered by the vaccine. HPV-16 and HPV-18 are expected to cover 70% of the women with severe disease (Widdice & Kahn, 2006). In the population studied here, the targeted vaccine types will only cover 55% of the women with high-risk infection seen at colposcopy. The true clinical implication will depend on the

amount of cross-protection the HPV vaccine will provide, which has been noted with HPV-31 and HPV-45 (Harper et al., 2006).

3.4.1 Future work

The most obvious opportunity to arise from this research is to use the clinical material collected for a retrospective cohort study and thereby determine the prognostic value of the molecular markers examined here. This approach would involve conducting a follow-up of nested case control studies of invasive cancer. This study has a large bank of nucleic acid extracted from cervical samples, that are stored at -70°C. Linkage can be used to identify future cases of cervical cancer from patients that have been enrolled here. Cases of cervical cancer or any other condition that may arise could trigger the original extract to be analyzed for the presence of HPV biomarkers. HPV DNA prevalence can then be compared to the corresponding prevalence in specimens of individuals from the same cohort who did not develop the condition under otherwise equivalent exposures. Such work could substantiate a recent finding that significant HPV oncogene transcript levels have an unfavourable prognosis in cervical cancer (de Boer et al., 2007).

Consequently, it is possible that further studies using a patient follow-up approach may find that RNA molecular tests (i.e. E6 RQ) are better for cervical cancer prognosis than diagnosis. Patients in this study could be candidates for a future prospective study to determine the risk of developing CIN and cancer. Such a study could be used to examine the prognostic value of HPV viral transcripts. However, given the mandate to treat patients with advanced

dysplasia, such a study would be restricted to the follow-up of patients with low-grade dysplasia or at least to patients that did not receive therapy such as LEEP.

The work presented here provides valuable information in light of the newly approved HPV vaccine. Worldwide, it seems that HPV-16 and HPV-18 currently account for around 70% of cervical cancers. However, after the implementation of the HPV vaccine, assuming a reasonable uptake rate, the prevalence of genotypes may have a good deal of geographical variation (Giles & Garland, 2006). It is expected that the introduction of the vaccine will reduce the number of women with cervical disease, but this is not proven and purely hypothetical (Villa et al., 2006). The reduction in cervical disease with the eradication of the two most common high-risk HPV genotypes is a reasonable assumption.

However, another scenario is the increase in prevalence of a non-vaccine high-risk HPV genotype such as HPV-31, which then goes on to cause the same amount of disease. This scenario is even more plausible given the risk of disease associated with HPV-31 as shown here. In any case, the prevalence of high-risk HPV types at colopscopy provides a good base-line to measure the effectivness of an HPV vaccine program for the eradication of disease. Of course, the implications of HPV vaccination will not be seen at colposcopy clinics for many years and perhaps decades given that the target populaton for the vaccine are 20 years younger than the average age of women who get referred for treatment at colposcopy. The first measures of vaccine efficiency will likely be done with a cohort of young women from the general population 5-10 years after

the vaccine is introduced. For this reason, a prevalence study to establish a base-line in this population should be done in Saskatchewan. The importance of local surveillance is shown in this study as world-wide trends do not always apply regionally.

Finally, the use of HPV DNA testing as a front-line cervical cancer screening tool or as a reflex test for ASCUS Pap smears (ideally using liquid-based cytology) is expected to play a larger role in diagnostics in the near future. Urine as a potential source of clinical specimen has many advantages for screening and may facilitate targeting the currently underscreened populations. It has been suggested that urine testing could be used to detect HPV (Daponte et al., 2006). The advantages of urine screening over swabs have already been proven for Chlamydia and gonorrhea testing (Chan et al., 1999; Chan et al., 2000a; Chan et al., 2000b).

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APPENDICES

Appendix A

CONSENT FORM – HPV RESEARCH

Purpose

You are invited to participate in a research study of cervical cancer screening techniques. We hope to learn the effect of viral proteins on the risk of abnormal cells progressing to cervical cancer. The investigators of this study will be specifically looking at how different types of Human Papillomavirus (HPV) develop within cells of the cervix and the relationship this may have with abnormal PAP smears. This study is designed exclusively for the study of HPV and its relationship to cervical cancer. This research will NOT be collecting human genetic information or releasing any of your DNA or information to any other database.

Confidentiality

The samples will be linked by a code and only your doctor will have the code. The researchers will not be able to identify you. Your sample will be analysed at the Saskatchewan Provincial Laboratory located at 3211 Albert St. Regina, SK and will only be identified by code. All samples will be securely stored at the laboratory and destroyed at the conclusion of the study. The study is anticipated to be completed by the Fall 2006. Patients have the right to request that their samples be destroyed at any time. At no time will personal identifying information be used in this study. Safeguards have been implemented to ensure patient confidentiality and anonymity.

Voluntary Participation

You were selected as a possible participant in this study because you have been scheduled for colposcopy in this clinic. Your involvement in this study is entirely voluntary and you may decline participation or withdraw at any time without affecting your current or future medical care. **If you wish to participate in this study, you must sign this form.** If you later decide to terminate your participation in this study, you should notify your doctor.

Risks and Benefits

The research requires a procedure that is performed during the PAP smear test – one additional swab is taken for research. This additional swab is very unlikely to present any risk for injury. Potential benefit may result from participation since HPV typing information is useful in some circumstances. These test results will be available to you through your doctor. The HPV detection and typing test results will only be generated by participation in this study. HOWEVER, WE CANNOT AND DO NOT GUARANTEE OR PROMISE THAT YOU WILL RECEIVE ANY BENEFITS FROM THIS STUDY.

For more information about the research, its findings or what will happen to your specimen contact either:	
N. Antonishyn, M.Sc. (Ph.D. student) - Principal researcher	787-7744
Dr. G. Horsman - Co-advisor, Medical Director Provincial Lab	787-8316
R. Kelln, Ph.D. - U of R faculty member supervising the research	585-4768

PLEASE ASK FOR EXPLANATION OF ANY INFORMATION THAT IS NOT CLEAR TO YOU.

If you have any questions about your rights as a subject participating in a clinical study, or if you would like to discuss your participation in the study, contact the Chair of Regina Qu'Appelle Health Region Research Ethics Board, at (306) 766-5451.

COLLABORATION OF

UNIVERSITY OF REGINA

Saskatchewan Health

Regina Qu'Appelle Health Region

PLEASE SIGN BELOW AND HAND BOTTOM PORTION TO YOUR PHYSICIAN DURING YOUR SCHEDULED EXAM.

.....(Tear here).....

CONSENT FORM – HPV STUDY

I have read the above information sheet and understand it. I will retain the top portion as my copy of this agreement.
I understand that my involvement in this study is entirely voluntary and that I may decline participation or withdraw at any time without affecting my current or future medical care.

_____
Personal Health Number

_____	_____	_____	_____
Signature of participant	Date	Signature of person obtaining consent	Date

Appendix B-1

Regina Qu'Appelle
HEALTH REGION

Certificate of Approval
Research Ethics Board

PRINCIPAL INVESTIGATOR	Mr. Nick Antonishyn University of Regina 3446 Westgate Avenue Regina SK S4S 1B5
APPROVAL DATE	August 26, 2003
RQHR PROJECT #	REB-03-50
TITLE	Correlation between HPV genotype, integration status and expression of HPV genes with the malignant transformation in cervical expholiate cell samples.

CERTIFICATION

The protocol and consent form for the above named project have been reviewed by the Regina Qu'Appelle Health Region Research Ethics Board and the experimental procedures were found to be acceptable on ethical grounds for research involving human subjects.

The Regina Qu'Appelle Health Region Research Ethics Board meets the standards outlined by Canada's Tri-Council Policy Statement for Ethical Conduct for Research Involving Humans.

Please note that all future correspondence regarding this project must include the RQHR project number.

Best wishes in your continuing research endeavours.

Dr. Elan Paluck, Chair
Regina Qu'Appelle Health Region
Research Ethics Board

/lgp
cc. Ms. C. Klassen, Corporate Services

This Certificate of Approval is valid provided there is no change in the experimental procedures. Any significant changes to the protocol must be reported to the Chair for the Board's consideration, in advance of implementation of such changes. You are required to provide a status report on an annual basis.

Please send all correspondence to: Research and Performance Support
Regina Qu'Appelle Health Region
2180 23rd Avenue, Regina SK S4S 0A5
Phone: (306) 766-5451 *Fax*: (306) 766-5530 *E-mail*: linda.picot@rqhealth.ca

Appendix B-2

UNIVERSITY OF REGINA

OFFICE OF RESEARCH SERVICES

Regina, Saskatchewan
Canada S4S 0A2
phone: (306)585-4775
fax: (306)585-4893
www.uregina.ca/research

DATE: June 19, 2003

TO: Mr. Nick Antonishyn
3446 Westgate Avenue
Regina, SK S4S 1B5

FROM: W. Wessel
Acting Chair, Research Ethics Board

Re: Correlation Between HPV Genotype, Integration Status and Expression of HPV Genes with the Malignant Transformation in Cervical Expholiate Cell Samples

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Please be advised that the University of Regina Research Ethics Board has reviewed your proposal and found it to be:

__/__ 1. ACCEPTABLE AS SUBMITTED. Only applicants with this designation have ethical approval to proceed with their research as described in their applications. The *Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans* requires the researcher to send the Chair of the REB annual reports and notice of project conclusion for research lasting more than one year (Section 1F). **ETHICAL CLEARANCE MUST BE RENEWED BY SUBMITTING A BRIEF STATUS REPORT EVERY TWELVE MONTHS.** Clearance will be revoked unless a satisfactory status report is received.

_____ 2. ACCEPTABLE SUBJECT TO CHANGES AND PRECAUTIONS (SEE ATTACHED). Changes must be submitted to the REB and subsequently approved prior to beginning research. Please address the concerns raised by the reviewer(s) by means of a supplementary memo to the Chair of the REB. Do not submit a new application. Please provide the supplementary memorandum, or contact the REB concerning the progress of the project, before **September 19, 2003** in order to keep your file active. Once changes are deemed acceptable, approval will be granted.

_____ 3. UNACCEPTABLE AS SUBMITTED. Please contact the Chair of the REB for advice on how the project proposal might be revised.

Warren Wessel

~~Paul Gingrich~~

c.c. R. Kelln, Supervisor

PG/sc/ethics2.dot

Appendix C-1

Trizol protocol version 2

1. vortex tube with brush for 15-30 seconds. (take right from dry ice)
2. transfer 1000 µl to yellow screw-capped eppie (pipet up and down before taking aliquot and have tubes on –20°C aluminium block)
3. spin @ **3000 x g (brake speed 7), 4°C, 5min**
4. remove supernatant with fine-tip bulb Pasteur pipet
5. add 800 µl of Trizol
6. vortex 15 sec (or 3 min at 5 speed with multieppie holder)
7. store at -70°C until extraction

---

8. add 160 µl chloroform, vortex and incubate RT for 5 min
9. spin @ **12,000 x g (brake speed 7), 4°C, 15min**
10. transfer upper phase (~400µl) to a fresh eppie
11. add 0.5 µl of 20µg/ml glycogen, mix and spin
12. add 400 µl of isopropyl alcohol, mix
13. spin @ **12,000 x g (brake speed 7), 4°C, 10min**
14. discard supernatant and wash pellet with 1 ml of 75% ethanol
15. spin @ **7,500 x g (brake speed 7), 4°C, 5min**
16. discard supernatant, spin and discard remaining supernatant
17. air dry 10 min
18. resuspend in 20 µl of a cocktail containing:

    15 µl RNase-free water
    1 µl RNAguard
    2 µl 10X DNaseI Buffer
    2 µl DNase I
19. incubate at 37°C for 30 min
20. add 130 µl of RNase-free water
21. add 800 µl of Trizol
22. vortex 15 sec (or 3 min at 5 speed with multieppie holder)
23. add 160 µl chloroform, vortex and incubate RT for 5 min
24. spin @ **12,000 x g (brake speed 7), 4°C, 15min**
25. transfer upper phase (~400µl) to a fresh eppie
26. add 0.5 µl of 20µg/ml glycogen, mix and spin
27. add 400 µl of isopropyl alcohol, mix
28. spin @ **12,000 x g (brake speed 7), 4°C, 10min**
29. discard supernatant and wash pellet with 1 ml of 75% ethanol
30. spin @ **7,500 x g (brake speed 7), 4°C, 5min**
31. discard supernatant, spin and discard remaining supernatant
32. air dry 10 min
33. resuspend in 50 µl DEPC water

Appendix C-2

Trizol protocol version 3

1. vortex tube with brush for 15-30 seconds. (take right from dry ice)
2. transfer 1000 µl to yellow screw-capped eppie (pipet up and down before taking aliquot and have tubes on –20°C aluminium block)
3. spin @ **3000 x g (brake speed 7), 4°C, 5min**
4. remove supernatant with fine-tip bulb Pasteur pipet
5. add 800 µl of Trizol
6. vortex 15 sec (or 3 min at 5 speed with multieppie holder)
7. store at -70°C until extraction

---

8. add 160 µl chloroform, vortex and incubate RT for 5 min
9. spin @ **12,000 x g (brake speed 7), 4°C, 15min**
10. transfer upper phase (~400µl) to a fresh eppie
11. add 800 µl of Trizol
12. vortex 15 sec (or 3 min at 5 speed with multieppie holder)
13. add 160 µl chloroform, vortex and incubate RT for 5 min
14. spin @ **12,000 x g (brake speed 7), 4°C, 15min**
15. transfer upper phase (~500µl) to a fresh eppie
16. add 0.5 µl of 20µg/ml glycogen, mix and spin
17. add 500 µl of isopropyl alcohol, mix
18. spin @ **12,000 x g (brake speed 7), 4°C, 10min**
19. discard supernatant and wash pellet with 1 ml of 75% ethanol (24 + 6)
20. spin @ **7,500 x g (brake speed 7), 4°C, 5min**
21. discard supernatant, spin and discard remaining supernatant
22. air dry 10 min
23. resuspend by gentle pipetting with 20 µl of a cocktail containing:

13 µl	RNase-free water	_____
1 µl	RNAguard	_____
2 µl	10X DNaseI Buffer	_____
2 µl	DNase I	_____

24. incubate at 37°C for 30 min
25. add 2 µl of 25 mM EDTA, mix and spin.
26. Incubate at 65°C for 10 min
27. add 180 µl of RNase-free water, mix spin and transfer to screw-cap tube
28. Store at -70°C