# Human papillomavirus (HPV) genotypes present in cervical biopsies with histologically confirmed high-grade dysplasia, and a negative Cobas high-risk HPV test result.

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## Abstract

The prevalence of cervical dysplasia and cancer has decreased ever since the introduction of cytology screening and recently, primary HPV testing. However, there have been incidences of histologically proven high-grade cervical dysplasia with a corresponding negative primary high-risk HPV test in New Zealand. Therefore, the first aim of the thesis was to determine whether HPV is in fact present in cases with histologically confirmed high grade cervical dysplasia and a corresponding negative high-risk HPV result from the Roche Cobas HPV test. The second aim was to then determine the HPV genotype and viral load present in the samples to help to determine if the Cobas testing identified the sample incorrectly as negative due to issues with assay sensitivity (viral load) or specificity (HPV genotype).

The results of the study initially identified the presence of low-risk HPV Type 6 DNA in three of the 16 samples. As a result, a qPCR assay was designed to select for HPV Type 6 to determine whether more of the samples contained HPV Type 6 DNA. It was found that seven samples contained only HPV Type 6 DNA, one contained only HPV Type 16 DNA and co-infections of both types 6 and 16 were identified in four of the samples. Three samples also contained a co-infection of either HPV Type 6 or 16 and an unknown HPV type. Quantification of HPV viral load was completed for the samples which ranged from $9.01 \times 10^5$ copies/µL to $8.18 \times 10^{10}$ copies/µL and it was determined that viral load did not correlate with CIN grade.

The research conducted in this thesis was the first New Zealand study to identify HPV Type 6 in samples containing high-grade cervical dysplasia. Further research will be required on the prevalence of HPV Type 6 in New Zealand and whether these samples may progress to cancerous lesions or if they will spontaneously regress.

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## Preface

My interest in HPV and microbiology began in high school when I completed the 162.101 Biology of Cells in my last year. The bacteria and virus component of the course sparked my interest, and my biology teacher at the time Mr Waters was in fact my supervisor Laryssa's husband. I then went into my Bachelor of Science thinking that I wanted to complete a post graduate course related to viruses. Luckily, I had Laryssa as my lecturer in my second year of my degree, where I nervously asked her if she would like to be my supervisor for a Masters. Even luckier, Rebecca, Laryssa's ex PhD student, had just completed her thesis on HPV in oropharyngeal cancer. Rebecca is the Lead Scientist in the Molecular laboratory at Medlab Central where she identified samples of histologically proven high-grade cervical dysplasia that had a corresponding negative high-risk HPV test. From there, the research commenced, where the samples were analysed and questions were answered, as well as new questions being formed.

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# Chapter 1 – Literature Review

## 1.1 Introduction

Human papillomavirus (HPV) is a small (~8000bp), non-enveloped DNA virus from the Papillomavirus family (Bzhalava et al., 2013). There are over 200 different HPV types which affect both men and woman (Bernard, 2005) and HPV is the most common sexually transmitted infection worldwide (World Health Organization, 2016). The different HPV types are categorised into different genotypes based on their L1 gene DNA sequence, where different genotypes have at least a 10% difference compared to any other HPV type (Gheit, 2019). HPV types are divided into high and low risk types based on their ability to cause cancer. High risk types include 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70. Low risk types include 6, 11, 42, 43 and 44 (Giuliano et al., 2008). HPV infects basal epithelial cells of the skin or inner lining of tissues and can be divided into two categories; cutaneous types and mucosal types (Giuliano et al., 2008). Cutaneous types target the skin of the hands and feet and mucosal types can infect the lining of the mouth, throat, respiratory tract and anogenital tract (Giuliano et al., 2008). Mucosal HPV types have a major role in the causation of cervical cancer (Wang et al., 2018) as well as other anogenital cancers and head and neck cancers (World Health Organization, 2019). HPV 16 and HPV 18 are the most prominent types of HPV that lead to cervical malignancy (Arbyn et al., 2020) and cause 70% of all HPV related cancers globally.

## 1.2 Characteristics of Human papillomavirus

Human papillomaviruses belong to the *Papovaviridae* family and the *Papillomavirus* genera. Papillomaviruses have icosahedral capsids which are composed of only protein (Brisson et al., 2020). Papillomaviruses viral genome is made up of circular double-stranded DNA (Munoz et al., 2006). *Papillomaviridae* have been shown to infect many species, including monkeys and bovines (IARC Working group on the Evaluation of Carcinogenic Risks to Humans, 2007). Human papillomaviruses infect only humans and can be divided into five evolutionary genotype groups (α, β, γ, μ, and ν). The Alpha genotype group is the largest and most studied group containing 64 HPV types (Van Doorslaer et al., 2012) it is responsible for 5% of all cancers worldwide (Egawa & Doorbar, 2017).

## 1.3 Gene expression

Most human papillomaviruses encode eight major proteins, six located in the “early” region and two located in the “late” region (Figure 1.1) . The “early” proteins (named E1, E2, E4-E6) play a role in genome replication and transcription, cell cycle, cell signalling and apoptosis control, immune modulation and structural modification of the infected cell (Fields et al., 1996).

Replication of the HPV viral genome requires only E1 and E2 proteins (Graham, 2017). The E1 protein is the only protein with enzymatic activity (Doorbar et al., 2012) and its main function is the regulation of HPV viral DNA replication. E1 has helicase activity and binds to

the HPV origin of replication prior to the initiation of DNA synthesis (Carter et al., 2011). The E2 protein is a sequence specific DNA binding protein with important functions in both viral replication and transcription (Uijterwaal et al., 2015). The precise role of E4 protein remains unclear, however it is associated with the collapse of cellular cytokeratin networks and this could facilitate viral release (zur Hausen, 1999). Due to E4 accumulating at later stages of infection it is more accurately described as a late protein (zur Hausen, 1999). E5 the smallest HPV oncoprotein, plays an important role in the HPV life cycle by delaying normal cell differentiation while maintaining cell cycle progression (DeFilippis et al., 2003). After expression of the early genes, the late genes as well as E4 are expressed in the upper layer of the epithelium, where the shed virus can now initiate new infections (Lie & Kristensen, 2008).

The “late” viral proteins (L1 and L2) are structural proteins that comprise the virus capsid required for virus transmission, spread and survival in the environment (Goodman, 2015).The icosahedral surface of the papillomavirus is formed singularly by the L1 protein (American Cancer Society, 2016) and can spontaneously assemble into virus like particles (VLPs) (American Cancer Society, 2016) which has been targeted to create vaccines for HPV (Parmin et al., 2019). Unlike L1, the L2 capsid protein lacks the capacity to form VLPs as it is only a minor capsid component (Hoste et al., 2013).

### 1.3.1 Oncogenic gene expression

E6 and E7 are identified as the major viral oncogenes (Yang et al., 2017) and are responsible for the malignant transformation of HPV-infected cells which can result in cancer (McLaughlin-Drubin & Münger, 2009). These oncoproteins modulate cellular proteins that regulate the cell cycle. Only the E6 and E7 viral proteins of high-risk HPV types have the ability to cause high-grade lesions and cervical cancer, based on their higher affinity to binding to the host cellular proteins. The E6 protein binds to the p53 tumour suppression protein and targets it for degradation (Tasoglu et al., 2015). The E7 protein binds to the retinoblastoma family of tumour suppression proteins resulting in the destabilization of these proteins and prevents repression of transcription of genes required for cell cycle progression (Tasoglu et al., 2015).

**Figure 1.1: The genomic organisation of HPV Type 16**. (Doorbar et al., 2012).

## 1.4 Life cycle

Papillomaviruses initial infection requires entry through micro abrasions/wounds in the epithelium to infect basal keratinocytes (Doorbar et al., 2012). The basal cell is the only cell that has the ability to proliferate, which is why the HPV lifecycle is tightly regulated by the differentiation process of the host cells (Kajitani et al., 2012). The infection initiates through the binding of the L1 major capsid protein to the heparan sulfate proteoglycan (HSPG) on the basal cell membrane (Kajitani et al., 2012). The virus then enters the cell surface on the basal membrane through interaction with the receptor expressed on the keratinocytes in the wound healing process (Kines et al., 2009).

The establishment phase then occurs where DNA replication which is independent of the cell cycle amplifies the viral copy number to around 50-100 copies per cell (Pinidis et al., 2016). A maintenance phase then follows, where genomic HPV DNA is transported into the nucleus and maintained at the 50-100 copies per cell (Moody & Laimins, 2010). It is believed that viral proteins E1 and E2 are expressed in the maintenance phase to sustain the viral DNA as an episome (Wilson et al., 2002) and to facilitate the correct segregation of genomes during cell division (You et al., 2004). The maintenance phase allows HPV to continue infection of the epithelial cells for a long duration while avoiding activation of the local immune response (Westrich et al., 2017). The HPV copy number is therefore maintained as the basal cells divide.

Once the basal cells are pushed to the suprabasal compartment, they lose their ability to divide and initiate the terminal differentiation programme. Papillomaviruses take advantage and replicate in this compartment and are released into the environment with the disintegration of the epithelial cells (Munoz et al., 2006). Free virus particles can survive for extended periods in the environment and normally reinfect cells adjacent to the site from which they were shed (Graham, 2017).

## 1.5 HPV related disease

Human papillomaviruses infect the mucosal or cutaneous epithelium and, in many cases, persist asymptomatically. The majority of HPV infections resolve spontaneously within two years (Flores-Miramontes et al., 2020). Papillomavirus genotype groups α, β, γ, μ, and ν can all cause cutaneous infections. Alpha genotype group also have genotypes which cause mucosal infections that can lead to HPV associated cancers (Doorbar et al., 2012). Alpha papillomaviruses mucosal types are further differentiated into high and low risk groups depending on the severity and persistence of disease the type causes (Bernard, 2005). Low-risk types are associated with anogenital warts, and recurrent respiratory papillomatosis (Brianti et al., 2017). High risk types are associated with cervical, penile, anal, vaginal, vulvar, and oropharanygeal cancers (Dunne & Park, 2013). The main difference between low-risk and high-risk HPV types lies within their ability to cause cancer. High-risk types have the ability to cause malignant transformation whereas low-risk types cannot. This is due low risk types oncoproteins (E6 and E7) having lower binding affinities for p16 suppressor protein and the retinoblastoma tumour suppressor protein, which makes them unable to alter the cell cycle and induce proliferation. (Yeo-Teh et al., 2018).

Table 1.1 HPV related disease and associated genotypes.

| **Type of disease** | **HPV genotypes associated** |
|---|---|
| Common warts | 2, 7 |
| Plantar warts | 1, 4 |
| Flat cutaneous warts | 3, 10 |
| Epidermodysplasia Verruciformis | Many types, predominately types 5 & 8 |
| Focal Epithelial Hyperplasia | 13, 32 |
| Genital warts | Many genotypes with 6 and 11 most common |
| Anogenital Malignancy | 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 |
| Cervical cancer | 16, 18 ,45, 31, 33, 52, 58 most frequently seen |
| Head and Neck cancers | Predominantly HPV 16 |

### 1.5.1 Benign HPV infections

HPV related disease and their associated genotypes are identified in Table 1.1. Cutaneous papillomavirus types are all classified as low risk types and are found in benign lesions such as warts. HPV induced cutaneous warts are benign and spontaneously regressing papillomas which usually regress within two years (Sterling et al., 2014). Individual cutaneous HPV types infect specific body sites, for example, HPV Type 1 infects the sole of the feet, and HPV Type 7 infects the hands (Norkin, 2010). The prevalence of cutaneous warts is as high as 20% and are found most frequently in children and adolescents (Jabłonska et al., 1997).

Cutaneous HPV types (predominately HPV Types 5 and 8) have also been identified in cases of Epidermodysplasia Verruciformis (EV), a rare disease which can progress to skin cancer through the development of large cutaneous warts (Majewski & Jabłońska, 1995). EV takes on average 24 years for the benign lesions to develop into skin cancer (Lutzner, 1978). Focal epithelial hyperplasia, also called Heck's disease, is a rare benign disease caused by HPV Types 13 and 32. HPV infects the oral mucosa and clinically presents itself as soft, distinct, mostly multiple, smooth papules or nodules (Bennett & Hinshaw, 2009; de Castro et al., 2016; Witkop Jr & Niswander, 1965). Heck's disease is associated with poverty and poor living conditions and is predominantly seen in Native American and the female youth of the Eskimo population (Bennett & Hinshaw, 2009).

HPV Types 6 and 11 are mucosal low-risk types and cause 90% of genital warts (Koutsky et al., 2002). Genital warts are spread through sexual contact, are highly contagious and develop within two-three months (Winer et al., 2005). The incidence of genital warts peaks between the ages 20-30 years, and 65% of those with an infected partner develop genital warts within eight months (Patel et al., 2013). Since the introduction of HPV vaccines, the prevalence of genital warts has rapidly decreased to the point where genital warts are hardly seen anymore, where only 1% of Australian women aged 21 years and under were found to have genital warts in 2011, compared to 10.5% in 2006 before the vaccination programme started (Ali et al., 2013).

## 1.6 HrHPV associated cancers

Viruses cause 15% of all human cancers, and nearly a third of these are attributable to HPV (Parkin & Bray, 2006). Persistent infection with a high-risk HPV type can lead to the development of cancer. HPV accounted for approximately 600,000 cases of cancer including cancers of the cervix, other anogenital sites and oropharynx globally in 2012 (Arbyn et al., 2012). Development of cancer is due to the interactions of the viral proteins E6 and E7. The E6 protein binds to the p53 tumour suppression protein and targets it for degradation (DeFilippis et al., 2003). The E7 protein binds to the retinoblastoma family of tumour suppression proteins resulting in the destabilization of these proteins and prevents repression of transcription of genes required for cell cycle progression (DeFilippis et al., 2003). Mucosal alpha types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are defined as high risk HPV types (hrHPV) by the World Health Organisation due to their high risk of

causing cancer (Munoz et al., 2006). Types 26, 53, 66, 67, 68, 70, 73 and 82 are named probable carcinogenic types due to rarely but consistently being identified in singular HPV infections, causing 3% of cervical cancer cases (Halec et al., 2014).

### 1.6.1 Non cervical cancers

HPV has been identified as a risk or causative factor for oropharyngeal, anal, vulvar, penile, and vaginal cancers. The annual number of non-cervical cancer cases is approximately 100,000 cases globally (Chaturvedi, 2010). HPV was the cause of approximately 63% of oropharynx cancers, 93% of anal cancers, 40% of penile cancers, 64% of vaginal cancers and 51% of vulvar cancers in the United States from 1998-2003 (Gillison et al., 2008). The reason for the high number of anal cancers caused by HPV is due to the biological similarities between anal cancer and cervical cancer, where oncogenic HPV types have an affinity for infecting the immature squamous cells of the cervix and anus, named the transformation zone (Brianti et al., 2017). Similarly, to cervical cancer, hrHPV Types 16 and 18 are the two most prevalent types in non-cervical HPV caused cancers (Syrjänen et al., 2012). Risk factors of non-cervical HPV cancers include lifetime number of sexual partners and smoking (Rubin et al., 2001; Smith et al., 2009).

### 1.6.2 Cervical cancer

Cervical cancer ranked 3$^{rd}$ in both incidence and mortality in the women globally, with 342 deaths per 100,000 women years in 2020 (Ferlay et al., 2021). Sub-Saharan Africa, Central America and South-Central Asia have the highest distribution of cervical cancer with Swaziland having the highest cases in 2018 with approximately 75 cases per 100,000 women-years. Compared to a developed country like USA, which has approximately 7 cases per 100,000 women-years (Arbyn et al., 2020).

Doctor Harold zur Hausen was the first person to hypothesise that cervical cancer was caused by HPV in 1972 (Mammas & Spandidos, 2015). His hypothesis was not widely accepted when first addressed but multiple studies have proven his hypothesis. The International Agency for Research on Cancer (IARC) conducted a study using 1000 histologically proven invasive cervical cancer cases from 22 different countries and using PCR found that HPV DNA was detected in 99.7% of the tumours (Bosch et al., 1992). These findings lead to the conclusion that HPV caused cervical cancer.

Cervical cancer usually arises from the cervical transformation zone (Schiffman et al., 2007). The cervical transformation zone is the area of columnar epithelium that transforms into squamous epithelium (Moscicki et al., 2006).The transformation zone is more sensitive to both HPV infection and the transformation actions of HPV (Stanley, 2010). Carcinogenesis occurs in the transformation zone due to HPV's inability to complete its life cycle, which results in the upregulation of HPV E6 and E7 viral proteins and deregulation of growth control and the build-up of mutations which in turn results in the development of cervical cancer.

Cervical carcinoma progresses from normal cervical epithelium through the development of low and high grade cervical intraepithelial lesions (CINs) (Olusola et al., 2019). CIN is the precancerous condition in which abnormal cells grow on the surface of the cervix. HPV DNA can exist as either episomal form or integrated into the host genome. Viral DNA is present as an episome in low grade CIN, whereas the integrated form is predominantly in high grade CIN and invasive tumours (Major et al., 2005). Integration of HPV DNA disrupts the expression of the E1 and E2 genes, which is then followed by an up-regulation of E6 and E7 gene transcription (Hudelist et al., 2004). The up-regulation of the E6/E7 viral oncogenes promotes deregulation of cell-cycle control, initiating the transformation and immortalization of HPV-infected cells (Jeon & Lambert, 1995). CIN1 is regarded as mild dysplasia and involves the lower third or less of the cervical epithelium. CIN1 is harmless and predominately returns to normal by itself. CIN2 and CIN3 are classified as high grade dysplasia progresses where CIN2 involves more than a third of the cervical epithelium and CIN3 involves the entire epithelium (Mello & Sundstrom, 2020). High grade dysplasia (CIN2+) is treated and screened for to prevent development into cervical cancer. However, it can be argued that CIN2 is an unnecessary point. Many CIN2 cases are caused by types which do not cause cervical cancer, and the regression rate is very high (Demarco et al., 2017).The main determinant of whether an HPV infection will progress to severe CIN or invasive cervical cancer is the HPV genotype causing infection (Mello & Sundstrom, 2020). HPV 16 and HPV 18 are the most prominent types of HPV that lead to cervical malignancy and these two genotypes cause 70% of all HPV related cancers globally (Wang et al., 2018). Types 45, 31, 33, 52, 58 and 35 follow HPV Types 16 and 18 as the most frequent types involved in the causation of cervical cancer (Munoz et al., 2006).

## 1.7 HPV Epidemiology

The most consistent risk factors for cervical cancer are a woman's lifetime number of sexual partners and her age at the initiation of sexual activity (Bosch et al., 1992). The frequency of HPV infections a woman will have in her lifetime correlate with the number of sexual partners she has at any given time. Infection by oncogenic HPVs is necessary but not a sufficient cause of cervical cancer, it is believed that co-factors together with HPV progress a cervical HPV infection to cervical malignancy (Castellsagué et al., 2002). A study in India found that low socio-economic status, high parity and the sexual behaviours of a woman's husband were important risk factors for cervical cancer (Franceschi et al., 2003). A two-fold association was reported between cigarette smoking and cervical cancer, where the association increased with the greater number of cigarettes smoked (Hildesheim et al., 2001). In the same study it was found that there was a significant association with oral contraceptives and cervical cancer in women who have had three or more pregnancies. This statement was also agreed with by Franceschi et al (2006), where they found that women who had five or more pregnancies and used contraceptives for more than five years had a 12-fold increased risk of squamous-cell carcinoma of the cervix. However, it can be argued that this is just a surrogate marker for sexual activity, where other studies reported that there was no causal relationship between oral contraceptives and high-grade dysplasia (Becker et al., 1994; Moreno et al., 1995; Muñoz et al., 1993).

There has also been evidence which suggests a role of hormones in cervical cancer development. Through multiple epidemiological and mouse model studies, it was found that estrogen and ERα (estrogen nuclear receptor) are required for cervical carcinogenesis (Chung et al., 2010). The role of estrogen in cervical cancer may also explain why contraception and high parity are considered co-factors in the incidence of cervical cancer.

Approximately 80% of cervical cancer cases occur in non-developed countries (Mandelblatt et al., 2002). The large disease burden in these less developed regions is largely due to lack of appropriate cervical screening (Garland et al., 2012). In contrast, South-eastern Asia and Southern Europe have been reported to have the lowest prevalence of cervical cancer due to organised and successful screening programmes. (De Sanjosé et al., 2007). South-Central Asia is a region of various and overlapping ethnicities which results in a vast variety of cultural lifestyle patterns, which is believed to be a reason for the high prevalence of HPV infection (Bhatla et al., 2008). In Sub-Saharan Africa especially Eastern Africa, early age at first marriage, marriage with older men, poor hygiene are all key factors for the high prevalence (Bayo et al., 2002). The absence or inability to attend screenings are also important factors in the less-developed countries (Bayo et al., 2002).

Genotypes prevalence can vary in different parts of the world. HPV Type 16 is the most common type in the majority of the world, whereas HPV Type 52 has been reported as the most prevalent type in Japan and Taiwan or a close second to HPV Type 16 (De Sanjosé et al., 2007; Sasagawa et al., 2001). The prevalence of HPV infections also differs in different age groups, where HPV infections are seen to be more prevalent in women less than 25 years of age, and prevalence decreases with increasing age in the majority of the world. (Franceschi et al., 2006). Nigeria and India have similar HPV prevalence across all ages, and both have very high rates of cervical cancer and mortality (Ferlay et al., 2001).

In some countries and cultures, a positive HPV infection carries negative moral connotations (McCaffery et al., 2003). Sex-related discussions are taboo topics in Asian culture, which inhibits parents from discussing sex and sexual health with their children (Wong et al., 2020). Particular cultures also do not agree with HPV vaccination, due to the normalization of premarital sexual activity (Dailey & Krieger, 2017), or parental belief that daughters are too young for sexual activity therefore too young for the HPV vaccination (Dorell et al., 2014).

### 1.7.1 Cervical cancer in New Zealand

In New Zealand, there are around 150 new diagnoses and 50 deaths from cervical cancer each year (Ministry of Health, 2019). Similar to the rest of the world, HPV Types 16 and 18 are the most prevalent in cervical lesions in New Zealand (Simonella et al., 2013). HPV Type 52 was reported to have a higher prevalence in New Zealand (19%) than other comparable studies in developed countries (10.2%) (Guan et al., 2012; Simonella et al., 2013).

Cervical cancer and mortality differ across the different ethnic groups in New Zealand. Maori women had an incidence rate of 10.6 and non-Maori women had a rate of 6.2 per 100,000 women in 2005 (Claridge, 2018). Maori women also had a mortality rate of 8.1 compared to non-Maori women who had 4.4 per 100,000 women (Murton, 2021). One of the reasons believed for the higher rate in Maori women compared to European women is the differences

in stage at diagnosis due to being under screened (Brewer et al., 2009), where it was reported that Maori women were 1.4 times more likely to be diagnosed with cervical cancer at a later stage than non-Maori women (Brewer et al., 2011). This is believed to be the result of the increase in socioeconomic inequalities of Maori and European ethnicities in New Zealand, resulting in widening inequalities of cancer mortality (The Descriptive Epidemiology Group of IARC, 2002). Another reason for underscreening in Maori women is due to the sense of embarrassment and shame associated with cervical screening (Adcock et al., 2019; Wihongi, 2000). Pacific women however, had similar diagnostic stages to European women, therefore the difference in the rates of Pacific and European women is linked to postdiagnosis factors, such as access to and uptake of treatment (Brewer et al., 2009).

The incidence of cervical cancer in New Zealand is among the lowest in the world, due to the introduction and stability of screening and vaccination programmes. The New Zealand National Cervical Screening Programme (NCSP) was established in 1990 (National Screening Unit, 2008). Death rates have dropped dramatically since the introduction of the NCSP. In 1991, 105 women died from cervical cancer and in 2014 the number had declined to 46 (Claridge, 2018). Cervical cancer also dropped from 8$^{th}$ to 17$^{th}$ highest cause of cancer mortality in women between 1988 and 2015 (New Zealand Health Information Service, 2008).

The Ministry of Health plan to change the NCSP from cytology screening every three years for women 20-69 years of age to primary HPV screening every five years from ages 25-69 in 2023, with the option of self-testing. This change is predicted to reduce cervical cancer by a further 12-16% and save 4-13% annually in programme costs (Lew et al., 2016). The greater sensitivity and longer duration of protection from a negative primary HPV test compared to cytology screening will provide better protection for New Zealand women who are screened less frequently (Ronco et al., 2014). Not everyone agrees with this change, where a New Zealand study estimated that the proposed HPV screening policy would increase the overall incidence of cervical cancer by 47% in New Zealand due to the both changing the screening test and the additional two years in between screenings. (Cox et al., 2019). However, this study used extremely bias sensitivity figures for HPV testing (87%) and cytology screening (93%), which is a high sensitivity for conventional cytology screening, where in other studies it has been reported between 55%-75% (Halford et al., 2010; Kripke, 2008; Kulasingam et al., 2002). Therefore, I believe the findings drawn from this study are bias and skewed.

## 1.8 HPV diagnostics

### 1.8.1 Cytology

Cervical cancer mortality has consistently declined since the 1940s, which can be largely contributed to cervical screening using cytology (Yang et al., 2018). Cervical precancerous lesions are well defined and provide a pattern of development of lesions (CIN1-CIN3) histologically and cytologically. This enables the ability to determine the severeness of a lesion through screening. Globally, one of the two most prominent techniques to detect the progress of HPV involves cytology screening. The Papanicolaou (Pap) smear was

introduced in 1943 and has reduced the incidence of cervical cancer and prevented premature deaths (Traut & Papanicolaou, 1943). There are two forms of Papanicolaou (Pap) cytology screening: conventional and liquid-based cytology (LBC). Conventional cytology involves cells from the cervix being spread onto a glass slide, whereas, LBC requires that cervical cells are put into a vial containing preserving fluid to produce a thin layer of cells before they are put on a slide (Meggiolaro et al., 2016). Cells are then stained and examined microscopically for cellular attributes and changes consistent with pre-cancer and cancer. LBC was introduced to increase the sensitivity and specificity of cervical screening. However, multiple results suggest that LBC showed no significant difference in the detection of CIN2+ but did detect more CIN1 or low grade lesions, leading to a lower positive predictive value (Celik et al., 2008; Davey et al., 2007; Siebers et al., 2009).

Cytology-based screening has proven to be successful in developed countries where cytology is quality controlled, and screening is performed in an organised setting (call-recall system). Therefore, cytology-based screening hasn't always been successful in low-resource countries (Meijer et al., 2009). Countries around the world have started to implement primary HPV testing as the main HPV testing replacing cytology screening, with Netherlands being the first to do so in 2017 (Rizzo & Feldman, 2018). This change has proven to be more cost-effective and decreased cervical cancer mortality and incidence compared to the previous cytology screening programme. However, colposcopy referrals increased by 172%, where many women who had CIN1 or less were being unnecessarily referred (Jansen et al., 2021).

### 1.8.2 Polymerase chain reaction (PCR)

PCR testing is the most sensitive technique for diagnosing HPV and allows testing on samples with fewer cells, poorer DNA and fewer viral copies (Evans & Cooper, 2004). There are a wide variety of different forms of PCR in the detection of HPV. PCR uses either consensus primers to detect a variety of HPV types DNA or type-specific primers to detect specific HPV type DNA (Mirghani et al., 2014). The major advantage of PCR testing for HPV is its high sensitivity, where numbers as low as one viral copy per cell can be detected (Westra, 2014). This high sensitivity also can be a disadvantage, where a relatively high incidence of false positive cases occur due to the inability of hrHPV infections to be distinguished from transient infections and those persistent infections that will develop into CIN2/+ (Agorastos et al., 2015).

#### 1.8.2.1 FDA approved devices

The Food and Drug Association (FDA) has approved of five PCR based HPV diagnostic techniques; Digene hybrid capture 2® (HC2), Cervista®, Roche Cobas 4800®, BD Onclarity® and Aptima HPV®. HC2® targets the full HPV genome, where as Cervista® and Cobas 4800® targets the L1 gene of HPV. Cervista® also targets the E6/E7 genes similarly to BD Onclarity® and Aptima HPV®. However, unlike the other devices, which target DNA, the most recently approved device Aptima HPV® targets the mRNA of the E6/E7

oncogenes. These tests have a number of uses including triage of women with low-grade or atypical smears, monitoring the success of treatment or a test of cure in those with previous high-grade disease, or use as a primary screening test (Binnicker et al., 2014).

The ATHENA trial, a large prospective study which consisted of over 40,000 women proposed that cervical cancer screening should consist of only primary HPV testing using FDA approved devices, with Australia and Netherlands being the first countries to do so (Wright Jr et al., 2012; Wright et al., 2015). Women who test negative for a primary HPV test for cervical HPV infection have a substantially lower risk of development of CIN2+ compared to women with a negative cytology result (Ronco et al., 2014). Studies have also shown that hr-HPV testing has a higher sensitivity (79-96% compared to 49-82%) and similar specificity to cytology (69-80% compared to 81-84%) when detecting high-grade lesions (Costa et al., 2015; McKenna & McMenamin, 2014; Pileggi et al., 2014). Decrease in financial costs and time will also occur due to reduced specialist referrals and colposcopies.

## 1.9 Vaccination

HPV vaccines have been proven to be safe and highly effective against vaccine-type infections and cervical precancerous lesions (Hartwig et al., 2017). Countries with a high vaccination rate >70% (predominantly developed countries) have seen a 73-85% reduction in HPV prevalence of vaccinated types, and a decline of 41-57% in high grade cervical lesions (Drolet et al., 2019). Gardasil and Cervarix were the first HPV vaccines produced and Gardasil4® was the first vaccine which gained FDA approval in 2004. Gardasil4® protects against HPV Types 16, 18, 6 and 11, Gardasil9 contains additional types 31, 33, 45, 52 and 58 and Cervarix targets only HPV Types 16 and 18 (Paz-Zulueta et al., 2018).

New Zealand introduced HPV vaccination in 2008 using the Gardasil4® vaccine on only females born in 1990 or later. In 2017 the vaccine was switched to the Gardasil9® vaccine which also included males aged 9-26 years old (Ministry of Health, 2016). Women who had at least one dose of the Gardasil4® vaccine prior to 18 years of age had a 25% lower prevalence of high-grade cytology and 31% lower incidence of high-grade cervical cytology when they were aged 20-24 years (Innes et al., 2020). More than 80% of unvaccinated individuals will become infected with HPV by age 45 years (Chesson et al., 2014) therefore it is important that communities are protected by vaccination.

## 1.10 Conclusion

In this review HPV and its role in cervical cancer has been discussed including its risk factors, prevention, and presence in New Zealand. It is very clear from the literature reviewed that HPV and cervical cancer are studied in great detail, with different ways of testing and diagnosing being produced constantly. With the primary screening programme for HPV testing in New Zealand proposed to change in 2023, more research is being completed to confirm the decisions being made are for the benefit of women in the protection and reduction of cervical cancer in New Zealand and globally.

## 1.11 Research question

There are two aims in the research presented. The first aim is to determine whether HPV is in fact present in 16 cases with histologically confirmed high grade cervical dysplasia (CIN2/3) and a corresponding negative high-risk HPV result from the Roche Cobas HPV test. The second aim is to determine the HPV genotype and viral load to help to determine if the Cobas testing identified the sample incorrectly as negative due to issues with assay sensitivity (viral load) or specificity (HPV genotype).

# Chapter 2 – Detection of HPV Type 6 CIN2/3 high-risk negative cases

## 2.1 Abstract

Cervical cancer is the third most frequent cancer in the world and is responsible for approximately 600,000 cases each year globally. High risk Human Papillomavirus (HPV) is associated with 99.7% of cervical cancer. Cases of HPV associated high-grade dysplasia and cervical cancer can be detected using molecular screening platforms. However, there is evidence that there are rare cases of high-grade dysplasia which are negative for HPV DNA in these molecular screening tests. Therefore, the aim of the study is to determine whether HPV is present in 16 cases with histologically confirmed high grade cervical dysplasia and a corresponding Cobas-negative high-risk HPV result. Biopsy samples were screened for the presence of amplifiable DNA and HPV types using molecular tools targeting the human beta-globin and HPV E6 and L1 genes. Positive beta-globin samples which also showed HPV amplification were sequenced to determine HPV genotype. Of the 16 samples tested, 15 samples had amplifiable beta-globin DNA. However, only three of the 12 samples that had amplifiable HPV L1 gene DNA could be successfully sequenced for the presence of HPV Type 6. The results of this study are the first time HPV Type 6 has been detected in samples of high-grade dysplasia with a negative Cobas test for high-risk HPV in New Zealand. Further work is needed to determine whether other samples contained the presence of HPV type 6 and if the HPV type 6 positive samples contained any co-infection with a high-risk HPV type of low levels.

## 2.2 Introduction

Human papillomavirus (HPV) infections are the most common sexually transmitted infection worldwide and almost all sexually active individuals will become infected with an HPV strain at some point in their lifetime (Dunne et al., 2007). The majority of HPV infections are benign and are resolved spontaneously (Burd, 2003). However, persistent infection with an oncogenic type of HPV may lead to precancerous lesions and cancer (Serrano et al., 2018). Each year, approximately 600,000 cases of HPV associated cancer occur worldwide, causing 8.6% and 0.8% of all cancers of women and men respectively (de Martel et al., 2017). High risk HPV types, such as HPV Types 16 and 18, are responsible for 70% of cervical cancers and a proportion of anogenital and oropharyngeal cancers. Women who develop cervical cancer are reported to have had the same high-risk HPV type detected 3-5 years prior to cancer incidence (Chan et al., 2019).

Screening techniques for cervical cancer have been implemented to efficiently and accurately identify those individuals who, are risk of developing invasive cancer. Cytological screening has a reporting system to distinguish low- and high-grade lesions. Briefly, smears are stained and examined microscopically for changes suggestive of precancer. Based on the observations, the cellular changes are reported as low grade, high grade or cancer. Patients who are suspected of having cervical lesions are also asked to undertake a cervical biopsy. The biopsies are examined histologically for cervical lesions which are graded CIN1-3. This correlates with a low-grade/high-grade approach of cytology as CIN1 is considered a low-grade lesion, while CIN2-3 are considered high grade lesions. CIN1 involves the lower third or less of cervical epithelium and CIN3 includes the full thickness of the epithelium

(Mello & Sundstrom, 2020). CIN2 is the intermediate category which is equivalent to moderate dysplasia but is also regarded as a high-grade lesion (Ismail et al., 1989). CIN1 are generally considered transient infections while CIN2-3 are considered severe persistent infections. CIN3 takes a long time to develop, approximately 15 to 20 years (Pietrangelo, 2019) and if left undetected and untreated, can progress to cervical cancer. The duration it takes for cervical cancer to develop allows for a long time for the precancerous lesions to be detected and treated through screening. The cytology screening been shown to be an effective method of detecting high-grade squamous intraepithelial lesions where conventional cytology has obtained a specificity and sensitivity of approximately 61-71% and 75% respectively (Kulasingam et al., 2002). Studies have shown that the mortality rate of cervical cancer would be 5.3 times higher in the absence of cytology screening or 65% lower if all women regularly screened at ages 50-64 (Landy et al., 2016).

Due to the established causal link between high risk HPV types and cervical cancer as well as the low sensitivity of cytology, the use of molecular diagnostic testing has been introduced as the primary screening method in some countries (for example Netherlands and Australia) (Wright Jr et al., 2012). Molecular diagnostic techniques for HPV, also known as hr-HPV testing, have shown to have a significant impact on being able to diagnose cervical cancer and precancerous lesions. The sensitivity of hrHPV testing (80-98%) (Kocken et al., 2012; Longatto-Filho et al., 2012; Stanczuk et al., 2016) outweighs that of cytology, at the cost of a lower specificity (Hoste et al., 2013). HrHPV testing has a 99% negative predictive value for CIN2+ lesions which enables women to extend their screening visits to five years compared to three years from cytology screening if negative results from hrHPV testing are given (Gage et al., 2014). One of the main limitations of hrHPV testing is the relatively high incidence of false positive cases due to the inability of being able to distinguish persistent hr-HPV infections that can develop into CIN2/+ lesions from transient infections (Agorastos et al., 2015).

The hrHPV testing used in New Zealand includes the Cobas 4800 (Roche Diagnostics, Switzerland) which features automated sample preparation combined with real time PCR technology in a single assay. The FDA approved molecular device identifies pooled high-risk oncogenic HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) as a collective, as well as HPV Types 16 and 18 individually (Heideman et al., 2011). The types are detected by probes with different reporter dyes to track the different targets in the multiplex reaction. The sensitivity and specificity of the Cobas 4800 for detecting CIN2+ is 71-99% and 24-86% respectively, depending on the population group studied (Binnicker et al., 2014; Cuzick et al., 2013; Heideman et al., 2011; Mesher et al., 2013; Phillips et al., 2015; Stoler et al., 2011; Szarewski et al., 2012). The Cobas 4800 has shown less cross-reactivity with low risk HPV types compared to other molecular diagnostic techniques (Cui et al., 2014).

However, internationally, there have been reports of cases of histologically confirmed CIN 2/+ high grade lesions testing negative for high-risk HPV DNA using the Cobas 4800 testing (Stoler et al., 2011). To further examine this issue, the ATHENA study evaluated the clinical performance of the Cobas 4800 HPV test for high-risk HPVs in American woman 21 years or older (Stoler et at., 2011), where it was found that 4.1% (1,923/47,208) of the cases showed the presence of abnormal cells in the cervical tissue. Among those women, 32.6% of them had hrHPV infections. Petry et al. (2016) analysed the ATHENA study for CIN2/3 positive and negative Cobas results, where 497 were

detected to have CIN2+, of which 55 of these tested negative for HPV. Additional testing using another platform, Amplicor (Roche Molecular Systems, USA), identified 58% of the 55 cases as positive for low-risk HPV types 73 and 82, and some co-infected with high-risk HPV Type 52. Low-risk HPV Types 73 and 82 are not included in the Cobas 4800 HPV test and it is believed that HPV Types 73 and 82 may need to be changed to high-risk types (Halec et al., 2014) due to their ability to rarely but consistently cause invasive cervical cancer. The ATHENA study was the first to show evidence that non high-risk HPV types can be present in cases of cervical dysplasia and cervical cancer. These findings were supported by Vasilyeva et al. (2021) who identified 16% (8/50) of American women tested negative for hrHPV for Cobas 4800 testing despite prior proven invasive squamous cell carcinoma.

In New Zealand, there are 150 new diagnoses and 50 deaths from cervical cancer each year (Ministry of Health, 2017). Despite a significant amount of HPV research over the last 10 years, there is no previous research regarding cases of high-grade cervical dysplasia with a corresponding negative Cobas hr-HPV test in New Zealand. Therefore, the specific aim of this research, is to determine whether HPV is in fact present in cases with histologically confirmed high grade dysplasia and a corresponding negative high-risk HPV result from the Cobas HPV test. The results of this study will identify if the HPV type/s causing high-grade cervical dysplasia are included in the Cobas assay. The identification of the type will identify if a high-risk or low-risk type is causing infection which may determine if the case will potentially progress to cervical cancer or if it will regress in the future. The addition of this specific information will help determine knowledge based on New Zealand parameters and not basing assumptions from international data. The findings drawn from this study will be helpful in New Zealand and worldwide due to the Cobas 4800 being a well-known and globally used testing platform.

## 2.3 Methods

*Ethics*

Ethics approval was obtained from the Health and Disabilities Ethics Committee. An Out of Scope form was completed and accepted on the 1st of October 2020.

*Sample collection/Case description*

Cases for this study were chosen through searches of 42-month review documents provided to laboratories by the National Cervical Screening Program between 2018 and 2020. Inclusion criteria were cases that had a histologically confirmed high grade lesion (CIN2+) and a negative Cobas hrHPV test within 6 months of the biopsy. Cases were limited to those which had both hrHPV testing and histology performed at MedLab Central, Palmerston North. A total of 16 samples were used for this study. Samples were collected as part of routine screening and follow-up. Samples were formalin fixed paraffin embedded biopsy samples.

*DNA extraction*

A block of paraffin embedded tissue was retrieved from the archive and a 10mm slice of the tissue block was used to complete DNA extraction. A pre-treatment for paraffin embedded tissue with Xylene was completed prior to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions for tissue. A final DNA extracted volume of 100uL was produced. The quality of extracted DNA was assessed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The extracted DNA was stored at Massey University (Palmerston North, New Zealand) at -20˚C for future use. A blank processing sample was also included, which contained DNA extraction reagents from the kit and no DNA sample, to ensure there was no contamination in the reagents. The blank processing sample was then used as an internal negative control for the real time (qPCR) and conventional PCR assays. A human cervical biopsy sample known to be positive for Beta-globin and HPV-16 was used as a positive control, with a water PCR blank used as a negative control for the various PCR assays.

*Beta-globin qPCR*

Beta-globin qPCR was used to detect the presence of amplifiable human DNA. Primers PCO3 and PCO4 (Table 2.1) were used to target the human beta-globin gene from the extracted DNA. Fast Start SYBR Green Master (Roche Diagnostics, Basel, Switzerland) mix was used in the 20uL reaction mix for detection of fluorescence in the qPCR along with 0.25uM of each primer and 25 ng/µL of extracted DNA. The beta-globin qPCR cycling conditions were 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds. The cycling conditions were then followed by a melt curve of 75 – 85˚C with 0.2˚C increments and a three second hold. A sample was considered positive if it crossed the threshold Ct value and produced a Tm of 82˚C ± 1.0˚C after the melt step.

*HPV 16 qPCR*

Samples that had a positive beta-globin qPCR were then tested to confirm the absence of HPV 16 qPCR. A 20uL reaction mix contained 0.25 µM of 16F and 16R primers (Table 2.1), 1X Fast Start SYBR Green Master (Roche Diagnostics) and approximately 50 ng/µL of sample DNA. qPCR cycling conditions were as follows; 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds, 62˚C for 30 seconds and 72˚C for 30 seconds. The cycling conditions were then followed by a melt step from 70-80˚C with 0.1˚C increments and a 5 second hold. A sample was considered positive if it crossed the threshold Ct value and produced a Tm of 79˚C ± 1.0˚C

*GP5/GP6 HPV conventional PCR*

GP5/GP6 PCR was used to amplify an HPV ~150bp L1 gene product for sequencing (Table 2.1). A 40uL reaction mixed contained 10uM of both GP5 and GP6 primers, 10x PCR Rxn buffer, 50mM $MgCl_2$, 1 unit of Platinum Taq polymerase (Thermo Fisher Scientific, Massachusetts, USA) and approximately 50 ng/µL of extracted DNA. The HPV conventional PCR cycling conditions are as follows, 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds, 49˚C for 30 seconds, 72˚C for 30 seconds. An additional 5 minutes was included at the final 72˚C elongation cycle. Samples were then visualised through a 2% agarose gel containing 1x SYBR Redsafe (Thermo Fisher Scientific) under UV light to confirm amplification.

*MY09/MY11 conventional PCR*

Amplification of a 450bp region of the HPV L1 gene was completed through conventional PCR for the purpose of sequencing. A 40µL reaction mix contained 0.2µM of MY09 and MY11 (Table 2.1), 0.3mM of each dNTP, 2mM $MgCl_2$, 1x PCR Reaction buffer, one unit of Platinum Taq polymerase (Thermo Fisher Scientific) and 25 ng/µL of extracted DNA. The HPV conventional PCR cycling conditions are as follows, 95˚C for 2 minutes, followed by 40 cycles of 95 ˚C for 5 seconds, 55 ˚C for 5 seconds, 72 ˚C for 30 seconds. An additional 7 minutes was included at the final 72 ˚C elongation cycle. Samples were then visualised through a 1.5% agarose gel containing 1x SYBR Redsafe (Thermo Fisher Scientific) under UV light to confirm amplification.

Table 2. 1 Genetic oligonucleotide primers used for DNA and HPV detection by Real-Time or Conventional PCR

| **Primer name** | **Primer sequence (5'-3')** | **Annealing temperature** | **Gene target/position** | **Product size (bp)** | **Reference** |
|---|---|---|---|---|---|
| PCO3 | ACA CAA CTG TGT TCA CTA GC | 55 ˚C | Human beta globin gene | 110 | (Saiki et al., 1988) |
| PCO4 | CAA CTT CAT CCA CGT TCA CC | | | | |
| 16F | GTG GAC CGG TCG ATG TAT GTC T | 62 ˚C | HPV 16 E6 | 209 | (Dictor & Warenholt, 2011) |
| 16R | TCC GGT TCT GCT TGT CCA GC | | | | |
| GP5 | TTT GTT ACT GTG GTA GAT AC | 49 ˚C | HPV L1 gene | 150 | (de Roda Husman et a 1995) |
| GP6 | GAA AAA TAA ACT GTA AAT CA | | | | |
| MY09 | CGT CGM ARR GGA WAC TGA TC | 55 ˚C | HPV L1 gene | 450 | (Gravitt et al., 2000) |
| MY11 | GCM CAG GGW CAT AAY AAT GG | | | | |

*M = A/C, W = A/T, Y = C/T, R = A/G

## 2.4 Results

The cytological and histological diagnosis of the 16 samples is presented in table 2.2 below. Six of the 16 samples had low-grade cytology, seven had high-grade cytology and three were negative. Nine of the 16 samples had moderate dysplasia (CIN2) and seven had severe dysplasia (CIN3)

Table 2.2 Cobas hrHPV testing, Cytological and histological results on 16 cervical samples used in this study

| Sample | HPV test | Cytology | Histology |
|---|---|---|---|
| 1 | Neg | Low grade | Moderate dysplasia (CIN2) |
| 2 | Neg | High grade | Severe dysplasia (CIN3) |
| 3 | Neg | High grade | Moderate dysplasia (CIN2) |
| 4 | Neg | Low grade | Moderate dysplasia (CIN2) |
| 5 | Neg | Negative | Severe dysplasia (CIN3) |
| 6 | Neg | High grade | Moderate dysplasia (CIN2) |
| 7 | Neg | High grade | Moderate dysplasia (CIN2) |
| 8 | Neg | Negative | Severe dysplasia (CIN3) |
| 9 | Neg | High grade | Severe dysplasia (CIN3) |
| 10 | Neg | Low grade | Moderate dysplasia (CIN2) |
| 11 | Neg | Negative | Severe dysplasia (CIN3) |
| 12 | Neg | Low grade | Moderate dysplasia (CIN2) |
| 13 | Neg | High grade | Severe dysplasia (CIN3) |
| 14 | Neg | High grade | Severe dysplasia (CIN3) |
| 15 | Neg | Low grade | Moderate dysplasia (CIN2) |
| 16 | Neg | Low grade | Moderate dysplasia (CIN2) |

The results of the PCR assays for the detection of DNA and HPV described above and sequencing analysis are summarized in Table 2.3 below. Following the beta-globin qPCR, 15 out of 16 samples showed the presence of amplifiable human DNA. The fifteen positive samples were hrHPV 16 negative, confirming the previous Cobas testing results. In order to determine if any genotype of HPV was present in these samples, the 15 samples were submitted to GP5/GP6 PCR to amplify a portion of the L1 gene. Twelve samples showed the presence of a positive amplicon at the expected size. However, after sequencing, none of the samples returned a clean sequence where the HPV source of the positive amplicon could be determined. Due to the lack of sequencing results, a second PCR (MY09/MY11) was used to amplify a portion of the L1 gene. Of the 15 samples tested, only three samples displayed a positive amplicon. After sequencing, the three samples returned a clean sequencing result from which BLAST analysis revealed that all three sequences had 100% sequence homology to the L1 gene of HPV 6 (GenBank DQ003079)

Of the three samples containing HPV type 6 DNA, one sample (#2) had cytology/histology results of high-grade/CIN3, while the other two samples (#6 and #12) had cytology/histology results of high grade/CIN2 and low grade/CIN2 (Table 2.2).

Table 2. 3: PCR assay and sequencing results from the DNA of the 16 clinical samples used in this study

| Sample | Beta Globin qPCR | GP5/GP6 | | MY09/MY11 | |
|---|---|---|---|---|---|
| | | Conventional PCR | Sequencing | Conventional PCR | Sequencing |
| 1 | Pos | Pos | No sequence | Neg | Neg |
| 2 | Pos | Pos | No sequence | Pos | Yes HPV 6 |
| 3 | Pos | Pos | No sequence | Neg | Neg |
| 4 | Pos | Neg | Not done | Neg | Neg |
| 5 | Pos | Neg | Not done | Neg | Neg |
| 6 | Pos | Pos | No sequence | Pos | Yes HPV 6 |
| 7 | Pos | Pos | No sequence | Neg | Neg |
| 8 | No | Not done | Not done | Not done | Not done |
| 9 | Pos | Pos | No sequence | Neg | Neg |
| 10 | Pos | Pos | No sequence | Neg | Neg |
| 11 | Pos | Pos | No sequence | Neg | Neg |
| 12 | Pos | Pos | No sequence | Pos | Yes HPV 6 |
| 13 | Pos | Neg | Not done | Neg | Neg |
| 14 | Pos | Pos | No sequence | Neg | Neg |
| 15 | Pos | Pos | No sequence | Neg | Neg |
| 16 | Pos | Pos | No sequence | Neg | Neg |

## 2.5 Discussion

There have been several studies that have examined the HPV types circulating in New Zealand women. A study in 2013 found 86% of tested women with a cervical cytology result of CIN2+ were positive for hrHPV (HPV Types 16 and 18) using linear array genotyping (Simonella et al., 2013). HPV Type 6 was not detected in any of these HPV positive cases. This was agreed with by another New Zealand study where none of the 418 CIN2/3 cases contained HPV Type 6 DNA using the same linear array genotyping method (Kang et al., 2015). Thus, the results of this study appear to provide the first description in New Zealand of the presence of HPV Type 6 in three cases of CIN2+ high-grade cervical dysplasia with negative Cobas results. Further work needs to be undertaken if the remaining 12 beta-globin positive DNA samples also contain HPV Type 6, although it appears that they may be positive for an HPV genotype given the positive band shown after the GP5/GP6 PCR, although sequencing was unsuccessful.

Human Papillomavirus Type 6 is one of the two most common HPV genotypes found in the mucosal epithelia, the other being HPV type 11 (Heinzel et al., 1995). HPV Type 6 was first identified as a causative agent in the formation of genital warts in 1981 (De Villiers et al., 2004) and now considered the etiological agents of at least 90% genital warts (Garland et al., 2009). HPV Type 6 has a lower binding affinity for the p53 and retinoblastoma tumour suppressor proteins and therefore classified as a low-risk HPV type due to their decreased ability to cause disease and malignancy (Barbosa et al., 1990). Occasionally, HPV Type 6 has been associated with benign hyperproliferative lesions of recurrent respiratory

papillomatosis (RRP) (Danielewski et al., 2013) as well as various malignancies, including Buschke-Lowenstein tumours, anal, vulval and penile carcinoma (De Villiers et al., 2004).

In a worldwide meta-analysis, HPV Type 6 was identified in 0.4% of cases of HPV-associated cervical dysplasia (Li et al., 2011). Another large-scale study reported that, five (0.06%) cases of HPV Type 6 were identified in women with cervical dysplasia in Costa Rica, four of which had CIN1 cytology and one with cervical cancer (Schiffman et al., 2007). Moreover, whilst HPV Type 6 was identified in three American studies, none of these studies found HPV Type 6 in CIN2/3 cases (Evans et al., 2002; Kong et al., 2007; Srodon et al., 2006). It should be noted that several studies failed to examine the presence of HPV co-infections therefore the HPV Type 6 positive cases could have been a co-infection with a high-risk type (Liao et al., 2020).

In a study by (Insinga et al., 2007) four common HPV types, low-risk Types 6, 11 and high-risk Types 16 and 18 were examined for their progression and regression in infections of young women over 36 months. It was found that HPV Type 11 never progressed past CIN1, however, 14% of HPV Type 6 infections progressed to CIN1 and 2% progressed to CIN2 after 36 months. However, none of the HPV Type 6 infections progressed to CIN3 over this period. In contrast, 21% and 10% of the hr-HPV Types 16 and 18 progressed to CIN1 in 36 months respectively, 9% and 6% to CIN2, and 7% and 3% to CIN3 in 36 months. While further studies are needed and the samples are currently blinded to their CIN grade beyond what was needed for selection into the study, it may be possible to conclude that the three HPV Type 6 patients in our study may have a low risk for the development of cervical cancer even though two of the samples were histologically proven CIN3. This is provided that no low-level co-infection with hr-HPV below the detection limit of the Cobas system is present.

The results of this study showed the presence of HPV Type 6 in two of the samples which had CIN2 lesions and another sample with CIN3 lesions. Whilst the presence of HPV Type 6 in the CIN2 samples is consistent with the findings of the studies described above, the finding of Type 6 in the CIN3 case appears to be unusual. Further testing is required to determine if a hrHPV co-infection is present in the CIN3 sample and whether other CIN3 cases in the study also have HPV Type 6 present and replication active within these lesions. It should be noted that presence of DNA of this common epithelial HPV Type alone does not conclude causality. In addition, the remaining nine samples which had amplicons using the GP5/6 L1 gene primers but were negative for sequencing and the MY09/MY11 L1 gene primers still need to be addressed. The reasoning for the negative sequencing and inconsistent L1 gene amplification results may lie with the PCRs themselves.

GP5/6 primers are reported to be more sensitive for detecting smaller viral loads because the amplification of the HPV L1 gene is 150bp compared to the MY09/MY11 primer set which is 450bp (Sotlar et al., 2004). The 150bp of the L1 gene may still have been amplified during the PCR reaction. However, due to the DNA being in formalin-fixed, paraffin embedded tissues (FFPE), this may have affected the success of the sequencing and larger fragment amplification. FFPE tissues enables the preservation of pathological tissues and allows for long term storage. However, formalin is a fixative, which leads to the

cross-linking of nucleic acids to proteins and other cellular constituents. This results in a difficult extraction of these analytes (Patel et al., 2017). Base purination and strand breaks can also occur due to age-related pH changes causing oxidation of the formalin (Groelz et al., 2013). This results in the integrity of the DNA potentially being poor and fragmented, which can produce unreliable results from PCR and sequencing.

This is the first study in New Zealand to identify HPV Type 6 in three cervical cases which are histologically confirmed CIN2/3 and have a corresponding negative Cobas test. However, further testing will be required to determine whether HPV Type 6 is causing the high-grade dysplasia by itself or included in a co-infection with a high-grade HPV Type. If it can be determined that HPV type 6 does not progress to CIN3+, and as CIN2 lesions not associated with HPV type 16 and 18 may regress, it may be possible to consider HPV Type 6 positive CIN2 cases as non-precancerous lesions. However, if in rare event HPV Type 6 associated lesions can be shown to progress in the absence of co-infection with a hrHPV, then HPV Type 6 may need to be accounted for when determining whether the lesion is severe and requires additional monitoring. The addition of this specific information will help determine knowledge based on New Zealand parameters for the use of Cobas 4800 platform.

# Chapter 3 Thesis – Development of qPCR for detection of HPV Type 6

## 3.1 Abstract

Three of the 16 samples with confirmed high-grade cervical dysplasia and a corresponding Cobas-negative high-risk HPV result from Chapter 2 returned with sequencing results for HPV Type 6. An additional 12 samples showed evidence of HPV DNA, but sequencing was unsuccessful. Thus, the aim of this study was to develop a qPCR for the detection of HPV Type 6 in the remaining 12 samples and to quantify the HPV viral load. Two sets of primers were selected using Geneious and were used in the screening of the presence of HPV Type 6 DNA through the development of a SYBR Green qPCR.

The results using the first set (6F87/6R171) showed that 100% (12/12) of the samples tested showed the presence of HPV Type 6 DNA by qPCR. However, the primers also amplified the HPV Type 16 control and the two HPV types could not be discerned by examining the melt curve. Therefore, the second set of primers were tested, and they were able to differentiate HPV Types 6 and 16 by melt curve analysis. When all 15 samples were tested, seven samples contained only HPV Type 6 DNA, one containing only HPV Type 16 DNA and co-infections of both types 6 and 16 were identified in four of the samples. Three samples also contained a co-infection of either HPV Type 6 or 16 and an unknown HPV type. Nine samples had sequence confirmation of the presence of the HPV Type 6 L1 gene DNA and the HPV viral load ranged from $9.01 \times 10^5$ copies/µL to $8.18 \times 10^{10}$ copies/µL. Although believed to be a positive relationship between viral load and severity of cervical lesion, there was no correlation found in the research.

The results found in this study are the first in New Zealand to identify and quantify HPV Type 6 in cases of high-grade cervical dysplasia and a Cobas negative result. Further work will be needed to determine whether lesions from the samples containing HPV Type 6 DNA have the potential to develop into a carcinoma or will regress.

## 3.2 Introduction

Worldwide, HPV Type 16 is the most prevalent HPV type seen in cases of cervical cancer followed by HPV Types 18 (19%), 31 (7%) and 33 (4%) (Harro et al., 2001).The same pattern is also seen in New Zealand (Sykes et al., 2014). As such, molecular testing using platforms such as Cobas diagnostics focuses on the detection of these high-risk HPV types in cervical cancer screening. However, there is growing evidence suggesting that molecular testing is missing the presence of HPV DNA in a small number of cases of high-grade cytology.

The ATHENA study (Petry et al., 2016) was the first study to identify and provide evidence of low-risk HPV types in histologically confirmed samples with a negative Cobas test. Out of 1,923 cases which showed the presence of abnormal tissue, 55 samples with CIN2+ tested negative for HPV. Low-risk HPV Types 73 and 82 were the most present of all the types in these samples. Preliminary results described in Chapter 2 also revealed the presence of a non-high-risk HPV type, HPV Type 6, in three cases of Cobas negative, high-grade cervical lesions. An additional 12 clinical samples also appeared to have the presence of an HPV type; however, sequencing and identification was not successful.

HPV Type 6 is a low-risk HPV type which is most often found in benign lesions such as genital warts (Sang & Barbosa, 1992). However, HPV Type 6 has previously been identified in several studies examining prevalence of HPV genotypes in cervical dysplasia, where HPV Type 6 is one of the most prevalent low-risk types in cervical cancer cases (0.4%) (Li et al., 2011; Schiffman et al., 2007).

Moreover, studies have shown that persistently high HPV viral load correlates with the progression and is a risk factor for cervical cancer (Josefsson et al., 2000). This has resulted in the progression of increased viral load being identified as a clinically useful predictive marker of infection. (Constandinou-Williams et al., 2010; Ylitalo et al., 2000).

Therefore, the aim of this research was to develop a real time PCR assay (qPCR) specifically for the detection and quantification of viral load of HPV Type 6. The qPCR was tested using the clinical samples previously described in Chapter 2 as being positive for HPV Type 6. The qPCR was then be used to screen the remaining 12 samples with unresolved HPV types and quantify the viral load in any HPV Type 6 positive samples. The results of this study may result in a stronger focus on HPV Type 6 in diagnostic testing, where HPV Type 6 could be tested for if a sample with histologically confirmed high grade dysplasia returns with a negative Cobas test. This could result in the reduction of unnecessary additional testing and financial costs if the sample can be determined to contain HPV 6 DNA.

## 3.3 Methods

Primer selection for the real-time PCR assay

The three HPV Type 6 L1 gene sequences detected in the clinical samples from Chapter 2 generated from the MY09/11 conventional PCR were aligned in Geneious (Biomatters, Auckland, New Zealand) and shown to have 100% nucleotide similarity. Appropriate primer locations to generate an amplicon between 90 and 200 base pairs were generated in Geneious and checked for specificity with HPV Type 6 using NCBI BLAST (National Center for Biotechnology Information, Maryland, USA) (Figure 3.1, Table 3.1). To confirm unique melt temperature in the qPCR, the expected melt curves were confirmed using uMelt (DNA-Utah, Utah, USA) The new forward and reverse primers were checked for successful amplification and specificity in the qPCR using the HPV Type 6 positive samples from Chapter 2 and a clinical sample previously identified as containing HPV type 16 (Lucas-Roxburgh, 2018).

HPV Type 6 L1 gene conventional PCR for sequencing confirmation

Amplification of a 106bp region of the HPV Type 6 L1 gene was completed through conventional PCR for the purpose of sequencing. A 40µL reaction mix contained 0.2µM of 6F87 and 6R171 (Table 3.1), 0.3mM of each dNTP, 2mM $MgCl_2$, 1x PCR Reaction buffer, one unit of Platinum Taq polymerase (Thermo Fisher Scientific, Massachusetts, USA) and approximately 25 ng/µL of extracted sample DNA. The HPV conventional PCR cycling conditions are as follows, 95˚C for 10 minutes, followed by 45 cycles of 95 ˚C for 15 seconds, 57 ˚C for 30 seconds, 72 ˚C for 30 seconds. An additional 7 minutes was included at the final 72 ˚C elongation cycle. Samples were then visualised through a 1.5% agarose gel

containing 1x SYBR Redsafe (Thermo Fisher Scientific) under UV light to confirm amplification. Samples with positive amplicons were prepared for sequencing, sequenced and analysed as described in Chapter 2.

HPV Type 6 L1 gene qPCR

The three HPV type 6 positive samples and the remaining 12 beta-globin positive clinical samples from Chapter 2 were subjected to the new qPCR as follows. A 20µL reaction mix contained 0.2µM of the Forward and Reverse primers (Table 3.1) and 1x Fast Start SYBR Green Master (Roche Diagnostics, Basel, Switzerland) and approximately 25 ng/µL of extracted sample DNA. Cycling conditions were as follows; 95˚C for 10 minutes, followed by 45 cycles of 95˚C for 15 seconds, 57˚C for 30 seconds, and 72˚C for 30 seconds. An additional 7 minutes was included at the final 72˚C cycle. PCR was followed by a melt curve from 70 - 90˚C with 0.2˚C increments and a two second hold.

Development of HPV Type 6 clones for quantification

To calculate the HPV Type 6 viral load in the clinical samples from the qPCR assay, standard curves were generated from a plasmid containing 450bp fragment from the HPV L1 gene from Sample 6 was amplified using the MY09/11 primers as described in Chapter 2. The PCR fragment was ligated using the pGEM – T Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions and transformed into *E.coli* JM109 high efficiency competent cells (Promega). Transformed bacteria were grown overnight at 37˚C on Luria-Bertani agar plates containing Ampicillin (100µg/ml Ampicillin sodium salt, Gibco®, Life technologies, New York, USA) and X-gal/IPTG (0.5mM IPTG, 40µg/mL X-Gal, Sigma-Aldrich, Missouri, USA) for blue/white selection. White colonies were selected and grown overnight at 37˚C in 5ml Luria-Bertani (LB) broth containing 100 µg/ml Ampicillin sodium salt (Gibco®). One millilitre of culture was centrifuged at 21,00xg for 10 minutes to pellet the bacteria. DNA was extracted using the DNeasy Extraction Kit (Thermo Fisher, Scientific) according to the manufacturer's instructions for Gram-negative bacteria. The resulting plasmid DNA was quantified using a Nanodrop 2000 (Thermo-Fisher, Scientific). qPCR was used to confirm the presence of the HPV Type 6 insert. To prepare dilution standards with known quantities of vector DNA copies, ten-fold standard dilutions of the vector DNA was prepared in DNase free water were made to 1 x $10^{-5}$ ng/µl (1.66 x $10^{4}$ copies of the target sequence/µl). Prepared dilutions were stored at 4˚C and consequently used within a day of preparation.

Quantification of HPV Type 6 viral load

Quantification of viral load was completed using the standard curves of the cloned sample 6 dilutions ($10^{2}$-$10^{-4)}$ and a known HPV 16 sample's beta-globin dilutions ($10^{1}$-$10^{-9}$). These standard curves were used to determine the number of copies of HPV copies/µL and beta-globin copies/µL in the samples. To determine the number of copies in the standard curve, the following formula was used:

$$\frac{Amount * 6.022x10^{23}}{length * 1x10^{9} * 660}$$

Where the amount is equal to the amount of DNA in the sample (ng) and length is the size of the total plasmid including the insert in base-pairs. The beta-globin copies/µL for the $10^1$ dilution was calculated as follows:

$$\frac{90 * 6.022x10^{23}}{3425 * 1x10^9 * 660} = 2.40\ x\ 10^{10} copies/\mu L$$

The total amount of genomic DNA in each sample was determined using the Nanodrop 2000. The same amount of each samples DNA (100ng) was then used with the respective qPCR standards to complete the "Beta-globin qPCR" method explained in Chapter 2 and the "HPV Type 6 L1 gene qPCR" described above, using primers 6F87 and 6R171, to calculate the number of beta-globin and HPV copies/µL. The HPV Type 6 viral load (copies/µL) of each sample was then calculated by dividing the HPV copies/µL by the beta-globin copies/µL. An efficiency greater than 0.94 was accepted for the use of the standard curve.

Table 3. 1. Genetic oligonucleotide primers generated for the development of an HPV Type 6 L1 specific qPCR

| **Primer name** | **Primer sequence (5'-3')** | **Product size (bp)** |
|---|---|---|
| 6F87 | GGA GTG GGC TTT TGA CAG GT | 106 |
| 6R171 | GGT TAT CGC CTC CCC CAA ATG G | |
| 6F106 | AGG TAA TGG CCT GTG ACT GC | 155 |
| 6R240 | TGT CTG CTG AAG TAA TGG CCT | |


Figure 3. 1 Primer locations of new HPV Type 6 L1 primers used to develop the qPCR assay for this study. The alignment generated from sequencing HPV amplicons of sample 6 and 12 using the MY09/11 primers (Chapter 2) and shows the locations of the new forward and reverse primers , 6F87/6R171 (red open arrows) and 6F106/6R240 (yellow open arrows)

## 3.4 Results

Initially, primers 6F87 and 6R171 were used to develop the HPV Type 6 specific qPCR. Blast analysis of the primers suggested that the two primers were specific for HPV Type 6. Umelt showed that the 106bp amplicon should generate a melt curve peak at 77.50˚C (±1.0˚C). As the BLAST results suggested that the primers were HPV Type 6 specific and the samples were supposed to be HPV Type 16 negative by Cobas analysis, a Umelt test was not done for HPV Type 16 and this primer set. Based on primer characteristics, the annealing temperature of 57 ˚C was selected. The known HPV Type 6 DNA sample (Sample 6) was then tested which yielded a positive result with a melt curve as expected with a peak at 77.67˚C.

To confirm specificity, a HPV Type 16 control from a known positive clinical sample (Lucas-Roxburgh, 2018) and the 12 beta-globin positive samples which produced no results from sequencing the L1 amplicons generated in Chapter 2 were then tested in the qPCR with the 6F87 and 6R171 primers. All 12 of the samples amplified in the qPCR assay and showed the presence of a melt curve consistent with HPV Type 6 DNA of the positive control sample (Sample 6). Unfortunately, the known HPV Type 16 control also showed the presence of DNA and a melt curve of 77.60 ˚C, suggesting the primers were not able to discriminate between the low-risk HPV Type 6 and the high-risk HPV Type 16 (Table 3.2).

However, as the 12 samples seemed to be able to be amplified with the 6F87/6R171 primers, the samples were reamplified using the same primers but in a conventional format to allow HPV sequence confirmation of the resulting amplification. Of the 12 samples sent for sequencing, 9 (75%) samples had successful sequencing results and returned clean electropherograms. Blast analysis revealed that the 9 samples had 100% sequence homology to the L1 gene of HPV Type 6 (GeneBank: DQ003079) (Table 3.2).

In order to improve the differentiation between HPV Types 6 and 16, the primer pair 6F140 and 6R206 were trialled. HPV Type 6 and 16 were distinguished by their melt points in the qPCR (78.50 ˚C compared to 85.70 ˚C respectively). A sample was considered positive for HPV Type 6 if it produced a $T_m$ of 78.50˚C (± 1.0˚C) and positive for HPV Type 16 if it produced a $T_m$ of 85.70˚C (± 1.0˚C). Following the 6F140/6R206 qPCR (Fig 3.2), HPV Type 6 DNA was detected in 47% (7/15) of the samples. HPV Type 16 was detected in 7% (1/15) and co-infections of both HPV Types 6 and 16 were also detected in 27% of the samples (4/15). Three samples (Sample 3, 7 and 11) showed evidence of either HPV Type 6 or 16, but also a melt peak not consistent with HPV types 6 or 16 (±1.0 ˚C) and was therefore considered an HPV co-infection that was unresolved.

Using the standard curves of beta-globin and one of the developed clones, HPV Type 6 viral load was calculated for 15 samples (Figure 3.3), which ranged from $2.95 \times 10^5$ copies/µL to $4.55 \times 10^{10}$ copies/µL. The average viral load was $3.08 \times 10^9$ copies/µL and the median was $7.62 \times 10^6$ copies/µL. The p-value to determine whether there was a difference in viral load in low-grade compared to high-grade lesions was 0.151.

A) B)

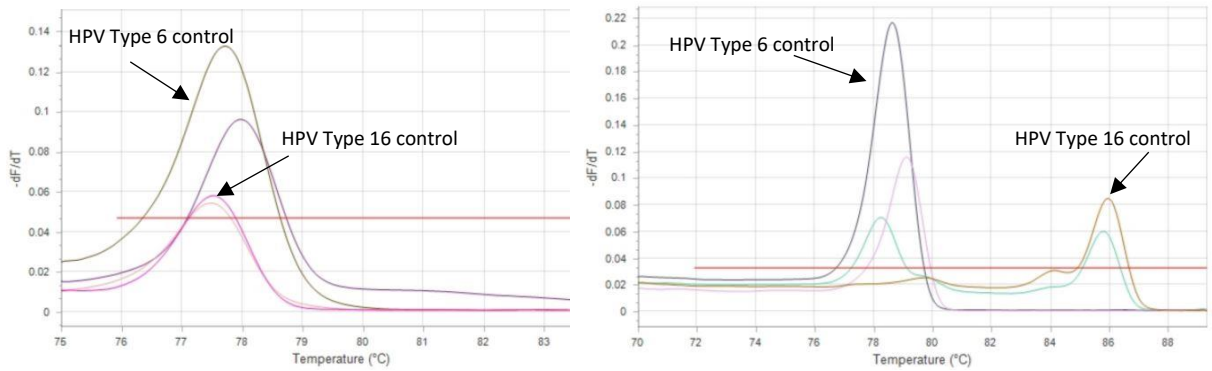


**Figure 3.2: 6F87/6R171 & 6F140/6R206 qPCR figure panel**. A) Melt curve of 6F87/6R171 qPCR. Positive samples shown with a $T_m$ of 77.70 ˚C ± 1.0˚C with no discrimination between HPV Types 6 or 16 B) Melt curve of 6F140/6R206 qPCR. Positive samples shown with $T_m$ of 78.50 ˚C ± 1.0 ˚C (Type 6) and/or $T_m$ of 85.50 ˚C ± 1.0 ˚C (Type 16).

A) B)

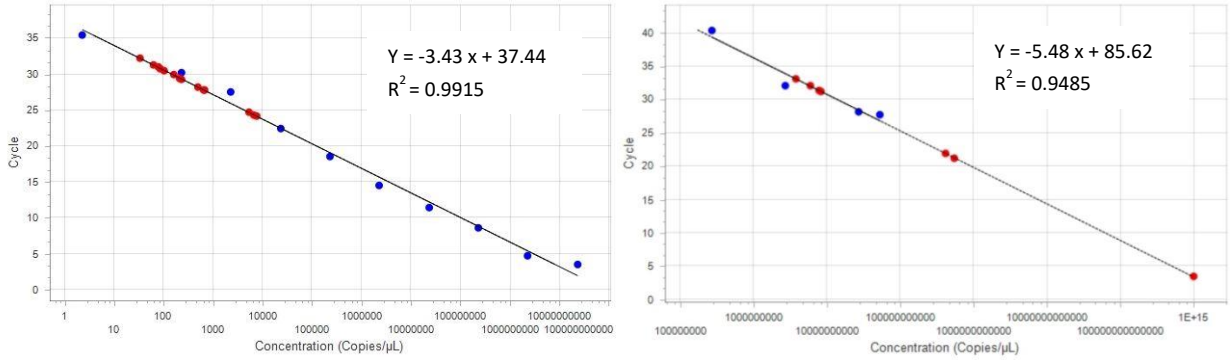


**Figure 3.3: Quantification of samples using standard curves figure panel**. A) Standard curve for the beta-globin gene. B) Standard curve for the developed HPV Type 6 clone.

Table 3. 2: HPV Type PCR assays and sequencing results from 16 samples

| Sample | 6F87/6R171 | | | 6F140/6R206 qPCR | | |
|---|---|---|---|---|---|---|
| | Conventional PCR | qPCR melt temperature (˚C) | qPCR probable HPV Type | Melt temperature (˚C) | Probable HPV type | Sequencing |
| 1 | Yes | 77.25 | 6 or 16 | 78.08 ˚C | 6 | Yes HPV 6 |
| 2 | N/A | N/A | N/A | 78.31 | 6 | Yes HPV 6 |
| 3 | Yes | 77.65 | 6 or 16 | 83.88, 85.79 | ? & 16 | No |
| 4 | Yes | 77.16 | 6 or 16 | 85.86 | 16 | No |
| 5 | Yes | 77.67 | 6 or 16 | 78.22, 85.79 | 6 & 16 | Yes HPV 6 |
| 6 | N/A | N/A | N/A | 78.47 | 6 | Yes HPV 6 |
| 7 | Yes | 84.59 | 6 or 16 | 79.72, 85.67 | ? &16 | Yes HPV 6 |
| 8 | Not done | Not done | Not done | Not done | Not done | Not done |
| 9 | Yes | 77.30 | 6 or 16 | 78.12, 85.60 | 6 & 16 | Yes HPV 6 |
| 10 | Yes | 77.51 | 6 or 16 | 78.28, 85.61 | 6 & 16 | Yes HPV 6 |
| 11 | Yes | 77.11 | 6 or 16 | 78.53, 84.20 | 6 & ? | Yes HPV 6 |
| 12 | N/A | N/A | N/A | 78.63 | 6 | Yes HPV 6 |
| 13 | Yes | 77.32 | 6 or 16 | 78.17 | 6 | No |
| 14 | Yes | 77.58 | 6 or 16 | 78.25, 85.79 | 6 & 16 | Yes HPV 6 |
| 15 | Yes | 77.39 | 6 or 16 | 78.25 | 6 | Yes HPV 6 |
| 16 | Yes | 77.25 | 6 or 16 | 78.28 | 6 | Yes HPV 6 |

? indicates an amplified product with a melt curve outside the range expected for HPV Type 6 or 16.

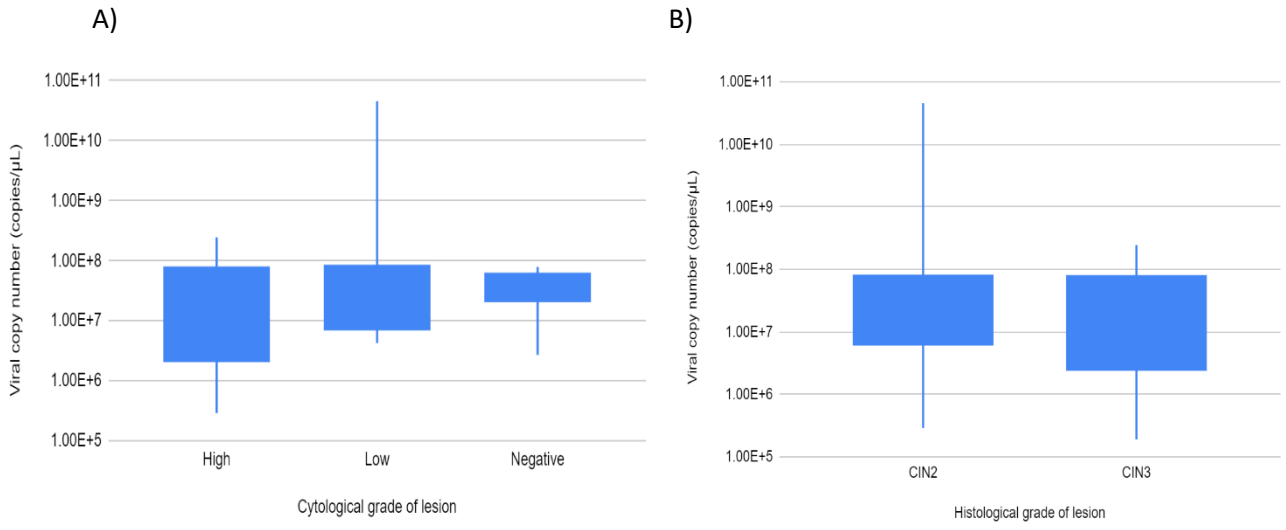


**Figure 3. 4**: Box and whisker graph of the HPV viral copy number (copies/µL) for the different cytology (A) and histological CIN grades of cervical dysplasia (B).

## 3.5 Discussion

The results of the sequencing from Chapter 2 from the three samples (#2, 6, 12) which determined the presence of HPV Type 6 produced the aim for this study. Unfortunately, the HPV type in the remaining 12 samples could not have been determined as the age of the sample and possible DNA degradation may have limited the sequencing success. However, as HPV Type 6 has been identified, the aim of Chapter 3 was to further examine the possibility of this HPV type in the remaining 12 samples via the development of a qPCR assay. The results then identified that 9 of the 12 samples contained HPV Type 6 by sequencing and three samples with no sequencing results are suggested as probable HPV Type 6 due to their results in the 6F87/6R171 qPCR being similar to the confirmed HPV Type 6 samples.

However, after completion of the 6F140/6R204 qPCR it was detected that one of the samples was infected with HPV Type 16, two of the samples had HPV Type 16 and a co-infection with an undetermined HPV type, four samples with a co-infection of both HPV Type 16 and 6, and one sample with a co-infection of HPV Type 6 and an undetermined type (Table 3.2). Of the samples which had a qPCR result of HPV Type 16, either as a sole HPV type or as a co-infection with HPV Type 6, had all previously returned a previous negative result for HPV Type 16 in the Cobas assay. This could suggest that a low threshold of HPV Type 16 DNA was unable to be detected by the Cobas testing and therefore should be considered false negatives. Further research will need to be completed on Samples 3, 7 and 11 which all showed an additional melt curve peak to determine what unknown HPV type is included in the sample.

Ge et al (2019) completed a very similar study where the findings drawn were also very similar. The American study identified 21 cases of high-grade dysplasia on a cervical biopsy and a negative Cobas hrHPV test. Of the 21 cases, two of them contained co-infections with a hrHPV type (16, 83, 84) and type 6. One of the cases had three negative consecutive Cobas tests. It was also found that a third of the samples contained an inadequate amount of HPV DNA to obtain a positive result of Cobas, which could imply that the threshold was not reached for DNA in a Cobas test. This may have also happened in this study, where the co-infections of Type 6 and 16 may have not contained enough HPV Type 16 DNA to be detected by the Cobas testing.

Furthermore, the three samples with the highest number of HPV copies/µL were also the three samples which displayed a positive amplicon in Chapter 2 and returned a positive sequencing result for the L1 gene of HPV Type 6. This agrees with previous literature, where a higher viral load correlates with a higher chance for HPV to be detected in the sample, and fluctuations in viral load below the detection threshold can lead to misclassification of samples to be HPV negative (Wu et al., 2006). Interestingly, Sample 12 which had the highest HPV viral load, had low grade cytology and moderate dysplasia (CIN2), which disagrees with literature where high HPV viral load is said to have a positive relationship with the severity of the lesion (Josefsson et al., 2000). However, no significant relationship

between viral load and severity of lesion can be concluded from the results in Figure 3.3 due to the p-value being 0.151.

Co-infection with multiple HPV genotypes is a common occurrence (Wu et al., 2013). Several studies have reported rates of multiple genotype concurrent infections ranging from 20%-36% (Beca et al., 2014; Castellsagué et al., 2012; Sohrabi et al., 2017). Cases of singular genotype infections have also seen to obtain additional genotypes through HPV infected sexual transmission (Vaccarella et al., 2011). A study found that HPV Type 16 was the most frequent hrHPV type in dual genotype infections and HPV Type 6 was the second most frequent low-risk HPV (lrHPV) type, following HPV Type 11 (Liao et al., 2020). A reason believed that lrHPV Types 6 and 11 are common in HPV co-infections is due to multiple transmission pathways, as they are responsible for laryngeal disease as well as genital infections (Muñoz et al., 2003). HPV Type 6 has been identified in co-infections in New Zealand, where it was found in 4% (2/49) of cervical cancer cases (Williamson et al., 2011).

The 6F140/6R204 primer set was produced after the 6F87/6R171 primer set so that HPV Type 6 was able to be selected for individually. HPV Type 6 isn't tested for in the Cobas 4800 testing and is thought to have a low chance to progress to cervical cancer. Histologically confirmed CIN2/3 cases which are negative for hrHPV could be further screened using the 6F140/6R204 qPCR to determine whether the case contains an HPV Type 6 infection, co-infection, or low-level HPV Type 16 infection below Cobas detection. The ability to determine if HPV Type 6 is only present could positively affect the patients and their wellbeing by creating a reduction on time and cost through the prevention of unnecessary referrals and colposcopy.

Due to the high positive predictive value of primary HPV testing, which results in many false positives cases and unnecessary invasive testing, HPV viral load has been considered as a diagnostic biomarker (Kim et al., 2020). HPV viral load can be used as a possible method for discriminating infections with underlying cervical disease (Schneider et al., 1997). It has also been found that increasing viral load has a causal relationship with severity of HPV disease (Dalstein et al., 2003) where high viral load of HPV DNA is associated with the risk of developing CIN (Josefsson et al., 2000) and low viral load was more prone to the clearing of the HPV infection (Dalstein et al., 2003). Using viral load as a diagnostic tool will also reduce the number of CIN2 overtreatment (Wang et al., 2013), where cases of CIN2 which will not develop into cervical cancer and will regress can be identified and no more additional testing would be necessary. The only study found which studied HPV type 6 viral load identified that the viral load of HPV type 6 was consistently higher in HPV 6 positive papilloma than in HPV 6 positive healthy laryngeal mucosa (Forslund et al., 2016).

Worldwide, HPV Type 6 has rarely been seen in high-grade dysplasia. However, with 12 (75%) of cases in this study being identified to contain HPV Type 6 DNA, this could suggest that the findings from this study are extremely unique compared to previous research. However, further research on the severity and potential cancer causation of the HPV Type 6 positive samples, the impact of low-levels of HPV Type 16 and the three cases containing non-HPV 6/16 identified in this research will need to be completed to determine whether the cases have the ability to progress into cervical cancer.

# Chapter 4 – Overall Discussion

## 4.1 Research Aims

There were two specific aims to this research. The first aim was to determine whether HPV is in fact present in 16 cases with histologically confirmed high grade cervical dysplasia (CIN2/3) and a corresponding negative high-risk HPV result from the Roche Cobas HPV test. The second aim was to determine the HPV genotype and viral load to help to determine if the Cobas testing identified the sample incorrectly as negative due to issues with assay sensitivity (viral load) or specificity (HPV genotype).

The results of this study answered both aims as HPV Type 6 and HPV Type 16 DNA, either individually or as co-infections, were found in 15/16 cases of histologically confirmed high grade dysplasia and a corresponding negative hrHPV Cobas result. Additionally, the HPV viral DNA ranged from $9.01 \times 10^5$ copies/µL to $4.55 \times 10^{10}$ copies/µL however there was no statistical significance between viral load and CIN grade of the cases. Furthermore, three samples appeared to contain a non-HPV type 6 or 16 genotype which could not be identified within the timeframe of this study.

## 4.2 Research impact

This was the first New Zealand study which identified HPV Type 6 DNA as a single infection or as a co-infection with a hrHPV Type (HPV Type 16) in high-grade CIN2/3 lesions and have a corresponding negative Cobas test. To date, only one other study has found similar results Ge et al. (2019). Therefore, the results provide a new insight into infection with low-risk HPV Type 6 and CIN2+ cases. However, further research is needed to fully understand the relationship between HPV Type 6 and whether its presence contributes to cervical dysplasia and progression to cancer.

Current knowledge suggests that HPV Type 6 rarely causes cervical cancer. Therefore, it can be inferred that the cases infected with HPV Type 6 in this study will most likely regress in the future and not become cancerous. Thus, a CIN2+ case with a negative Cobas test would not be a cause for concern and would not need further testing. The more significant question is the detection of what appears to be low levels of HPV Type 16. The Cobas test has a pre-set threshold of which cycle number the HPV should be detected by (Duan et al., 2020). The higher the viral load, the earlier the DNA will be detected in the PCR. If the HPV DNA in the sample is not picked up by the certain cycle number, then it is deemed negative. Studies have shown that even a low number of HPV Type 16 in CIN2+ cases may result in progression (Andersson et al., 2005). Therefore, a testing algorithm using other hrHPV testing platforms (BD Onclarity®, Cervista® and Aptima HPV®) may improve the sensitivity of detecting low levels of HPV Type 16 and allow more confidence the false negative cases are limited.

The uptake of early screening tests, such as PAP smears, and secondary colposcopies and HPV genotyping have gone a long way to improve the outcomes for women with CIN2+ cases of cervical dysplasia (Khan et al., 2017; Traut & Papanicolaou, 1943; van Hamont et al., 2006). Thus, limiting cases of false negatives and developing a better understanding of

the role of HPV Type 6 is important as research has shown that women participating in HPV testing can have adverse psychosocial responses (Bennett et al., 2019; O'connor et al., 2014).

## 4.3 Future research

A review of all the characteristics of the cases for the potential detection of any patterns would be a good way to start future research. This includes ethnicity, age, socioeconomic status, location and marital status. This may determine if HPV Type 6 is predominantly located in specific locations. It may also identify if HPV Type 6 is potentially transferred through sexual partners to cause infection and if seen more in married individuals or individuals who may have more sexual partners. If any patterns or relationships are found with the cases this may give an indication to why these samples were identified to contain HPV Type 6 DNA over other low-risk HPV types.

The cases in this study should also be submitted for p16 immunohistochemistry. An active high-risk HPV infection results in overexpression of the p16 tumour suppressor protein. This is caused by the HPV E7 protein inactivating the retinoblastoma pathway (Lewis, 2012). The E7 proteins encoded by low risk HPV types differ compared to the hrHPV types and as a result, p16 accumulation doesn't occur in cases infected by low risk HPV types (Genovés et al., 2014). This allows for p16 immunohistochemistry to be a selective marker for HPV infected cells which could result in cervical cancer. In a similar study to this research, Geibler et al. (2013) conducted p16 staining on 14 HPV samples where 13 of the 14 samples were infected by HPV 16 and all their tumours overexpressed p16. The remaining sample contained HPV 6 DNA, which tested negative for overexpression of p16. Completion of p16 immunohistochemistry on the samples used in this study will determine if the cases contained cancer causing cells, or if the infections are benign lesions with no potential progression to cervical cancer.

Although there have been several studies investigating the prevalence of hrHPV types (Clifford et al., 2006; Li et al., 2011; Smith et al., 2007), to date there have been no studies in New Zealand investigating the prevalence of HPV Type 6 in cervical samples. If there is a higher prevalence of HPV Type 6 infections compared to other countries, this could determine the reasoning for the number of HPV Type 6 infections found in this research. Similarly, as transmission of HPV occurs not only via sexual contact but also oral contact (Sánchez-Vargas et al., 2010) and it is believed that the oral cavity may serve as a natural reservoir for HPV outside the genital region (Giraldo et al., 2006). It has also been reported that HPV Type 6 are found in a high percentage of benign oral lesions (De Villiers, 1994). Therefore, studies to determine if HPV Type 6 is present in oral samples and oral sex as a possible transmission pathway could also be beneficial.

Research on the co-infections of HPV Types 6 and 16 would also help further the findings on the research in this study. Identifying the reason for the HPV Type 16 DNA samples to obtain a negative Cobas result and identifying the ability of these co-infections to cause cervical cancer may improve future implications and testing. It has been reported that

women with multiple infections were at increased risk of CIN2+ compared to those with single HPV infections (Chaturvedi et al., 2011). Determining this risk of cervical cancer may employ more intensive focus on co-infections with HPV Types 6 and 16.

The generalizability of the results in this study is partially limited by the sample size. A larger pool of samples from different regions of New Zealand would also help further this research. This allows the results from the research to not be specific to Medlab Central but all New Zealand.

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# Appendices

## Appendix 1 – HDEC Out of Scope Ethics Form

Health and Disability Ethics Committees

Health and Disability Ethics Committees
Ministry of Health
133 Molesworth Street
PO Box 5013
Wellington
6011
0800 4 ETHICS
hdecs@moh.govt.nz

1 October 2020

Mr Taylor Monk
Massey University
[redacted]

Dear Mr Monk,

| | |
|---|---|
| Study title: | HPV types present in cervical biopsies with histologically confirmed high grade dysplasia, and a negative cobas high risk HPV test result. |

Thank you for emailing HDEC a completed scope of review form on 13 September 2020. The Secretariat has assessed the information provided in your form and supporting documents against the Standard Operating Procedures.

Your study will not require submission to HDEC as, on the basis of the information you have submitted, it does not appear to be within the scope of HDEC review. This scope is described in section three of the Standard Operating Procedures for Health and Disability Ethics Committees.

An observational study requires HDEC review only if the study involves more than minimal risk (that is, potential participants could reasonably be expected to regard the probability and magnitude of possible harms resulting from their participation in the study to be greater than those encountered in those aspects of their everyday life that relate to the study).

For the avoidance of doubt, an observational study always involves more than minimal risk if it involves one or more of the following:

- one or more participants who will not have given informed consent to participate, or
- one or more participants who are vulnerable (that is, who have restricted capability to make independent decisions about their participation in the study), or
- standard treatment being withheld from one or more participants, or
- the storage, preservation or use of identifiable human tissue without consent, or
- the disclosure of identifiable health information without authorisation.

If you consider that our advice on your project being out of scope is in incorrect please contact us as soon as possible giving reasons for this.

This letter does not constitute ethical approval or endorsement for the activity described in your application, but may be used as evidence that HDEC review is not required for it.

Please note, your locality may have additional ethical review policies, please check with your locality. If your study involves a DHB, you must contact the DHB's research office before you begin. If your study involves a university or polytechnic, you must contact its institutional ethics committee before you begin.

Please don't hesitate to contact us for further information.

Yours sincerely,

Tristan Katz
Advisor
Health and Disability Ethics Committees
hdecs@moh.govt.nz

# Appendix 2 - Beta-Globin qPCR SOP

| **Beta-Globin qPCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | PC03 | ACA CAA CTG TGT TCA TCA CTA GC | 110 | 827-847 | Human Beta-globin gene |
| Reverse | PC04 | CAA CTT CAT CCA CGT TCA CC | | 917-937 | |

[1] Based on Human Beta-globin gene sequence (Genbank accession number: L26478)

**PCR Kit: Roche Faststart SYBR Green mastermix**

| **Reagent mix** | **Volume (20µL)** |
|---|---|
| Sterile distilled water | 8µL |
| 2x SYBR green mastermix | 10µL |
| 10 µM PC03 primer | 0.5µL |
| 10µM PC04 primer | 0.5µL |
| DNA | 2µL |

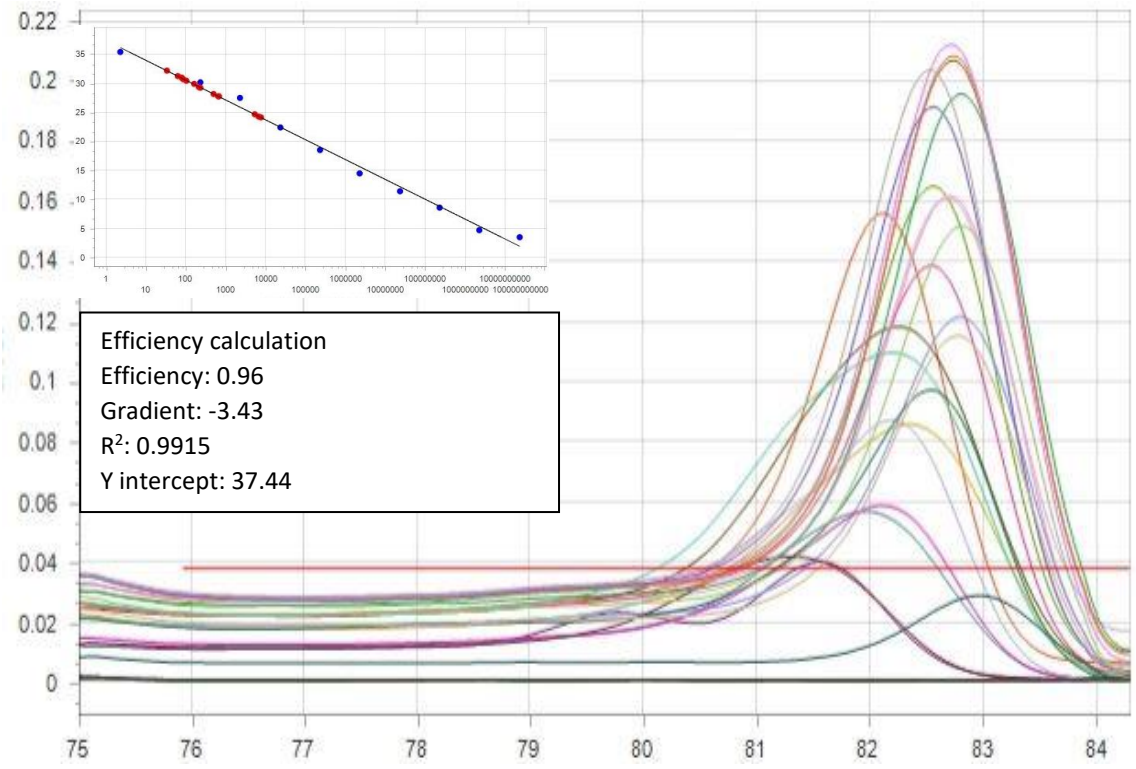
| **PCR controls** | **Description** |
|---|---|
| Positive | Beta-globin clone control (Plasmid with a 665 bp fragment of the human Beta-globin gene). |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** Beta-globin

| **Cycling Parameters** | **Temp (˚C)** | **Time** | **No. Cycles** |
|---|---|---|---|
| Hold | 95 | 10 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>55<br>72 | 15 sec<br>30 sec<br>30 sec | 40 |
| Melt | 75-85 (0.2 increments) | Three second hold | 1 |

| Electrophoresis | Description | Size of amplicon(s) (bp) |
|---|---|---|
| Agarose gel | 1.5% | 110 |
| MW marker | 100bp | |

**Standard curve and melt images**

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# Appendix 3 - HPV 16 qPCR SOP

| **HPV 16 qPCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | 16F | GTG GAC CGG TCG ATG TAT GTC T | 209 | 496-519 | HPV 16 E6/E7 gene |
| Reverse | 16R | TCC GGT TCT GCT TGT CCA GC | | 685-704 | |

**PCR Kit: Roche Faststart SYBR Green mastermix**

| **Reagent mix** | **Volume (20µL)** |
|---|---|
| Sterile distilled water | 8µL |
| 2x SYBR green mastermix | 10µL |
| 10 µM 16F primer | 0.5µL |
| 10µM 16R primer | 0.5µL |
| DNA | 2µL |

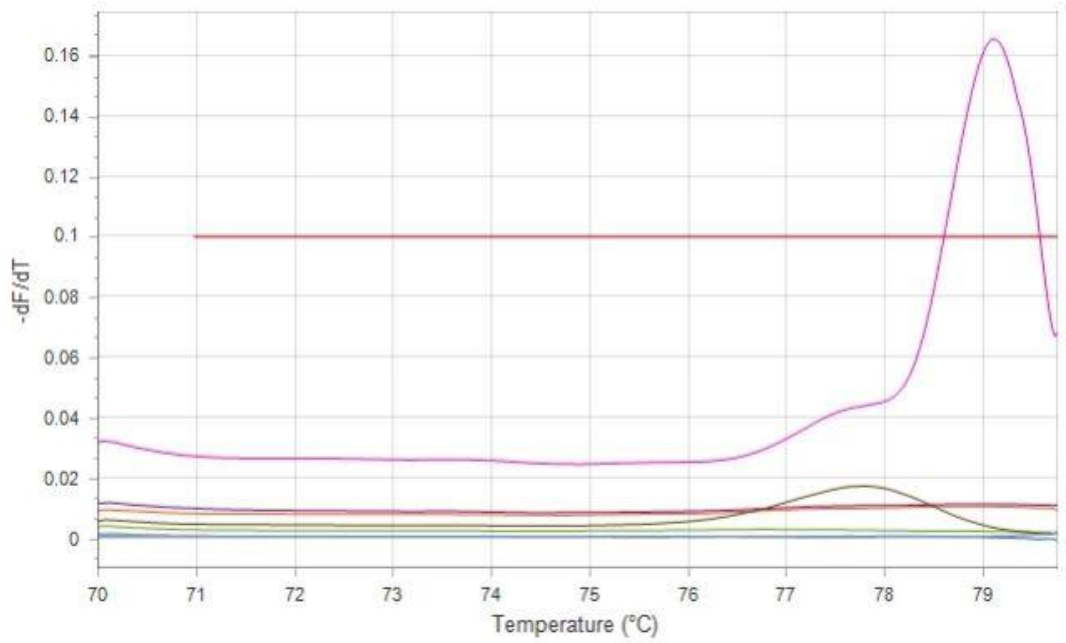
| **PCR controls** | **Description** |
|---|---|
| Positive | 16-1 clone control (Plasmid with 1.8kb fragment of the HPV 16 genome). |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** HPV 16

| **Cycling Parameters** | **Temp (˚C)** | **Time** | **No. Cycles** |
|---|---|---|---|
| Hold | 95 | 10 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>62<br>72 | 15 sec<br>30 sec<br>30 sec | 40 |
| Melt | 75-85 (0.2 increments) | Three second hold | 1 |

| Electrophoresis | Description | Size of amplicon(s) (bp) |
|---|---|---|
| Agarose gel | 1.5% | 209 |
| MW marker | 100bp | |

**Melt image**




**References**

Dictor, M., & Warenholt, J. (2011). Single-tube multiplex PCR using type-specific E6/E7 primers and capillary electrophoresis genotypes 21 human papillomaviruses in neoplasia. Infect Agent Cancer, 6(1), 1. doi: 10.1186/1750-9378-6-1

# Appendix 4 - GP5/6 PCR SOP

| **GP5/6 conventional PCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | GP5 | TTT GTT ACT GTG GTA GAT AC | 150 | 6624-6646 | HPV L1 gene |
| Reverse | GP6 | GAA AAA TAA ACT GTA AAT CA | | 6724-6746 | |

[1]Based on HPV 16 sequence (Genbank accession number: KO2718)

**PCR Kit:** Invitrogen Platinum Taq Polymerase

| **Reagent mix** | **Volume (40µL)** |
|---|---|
| Sterile distilled water | 27.4µL |
| 10x PCR buffer | 4µL |
| $MgCl_2$ | 1.6µL |
| dNTPs | 1.2µL |
| 10 µM GP5 primer | 0.8µL |
| 10µM GP6 primer | 0.8µL |
| Taq Polymerase | 0.2µL |
| DNA | 4µL |

| **PCR controls** | **Description** |
|---|---|
| Positive | Known positive clinical sample for HPV type 16 |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** GP5/6

| Cycling Parameters | Temp (˚C) | Time | No. Cycles |
|---|---|---|---|
| Hold | 95 | 10 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>49<br>72 | 15 sec<br>30 sec<br>30 sec | 40 |

| Electrophoresis | Description | Size of amplicon(s) (bp) |
|---|---|---|
| Agarose gel | 1.5% | 209 |
| MW marker | 100bp | |

**References**

de Roda Husman, A. M., Walboomers, J. M., van den Brule, A. J., Meijer, C. J., & Snijders, P. J. (1995). The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. The Journal Of General Virology, 76 ( Pt 4), 1057-1062.

# Appendix 5 - MY09/MY11 PCR SOP

| **MY09/11 conventional PCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | MY09 | CGT CCM ARR GGA WAC TGA TC | 450 | 7015-7034 | HPV L1 gene |
| Reverse | MY11 | GCM CAG GGW CAT AAY AAT GG | | 6583-6602 | |

**PCR Kit: Roche Faststart SYBR Green mastermix**

| **Reagent mix** | **Volume (20µL)** |
|---|---|
| Sterile distilled water | 25.4µL |
| 10x PCR | 4µL |
| $MgCl_2$ | 1.6µL |
| dNTPs | 1.2µL |
| 10 µM 16F primer | 0.8µL |
| 10µM 16R primer | 0.8µL |
| Taq polymerase | 0.2µL |
| DNA | 6µL |

| **PCR controls** | **Description** |
|---|---|
| Positive | Known positive clinical sample for HPV type 16 |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** HPV 16

| **Cycling Parameters** | **Temp (˚C)** | **Time** | **No. Cycles** |
|---|---|---|---|
| Hold | 95 | 2 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>55<br>72 | 5 sec<br>5 sec<br>30 sec | 40 |

| **Electrophoresis** | **Description** | **Size of amplicon(s) (bp)** |
|---|---|---|
| Agarose gel | 1.5% | |
| MW marker | 100bp | |

| **References** |
|---|
| Gravitt, P. E., Peyton, C. L., Alessi, T. Q., Wheeler, C. M., Coutlee, F., Hildesheim, A., ... & Apple, R. J. (2000). Improved amplification of genital human papillomaviruses. *Journal of clinical microbiology*, *38*(1), 357-361. |

# Appendix 6 - 6F87/6R171 qPCR SOP

| **6F87/6R171 qPCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | 6F | GGA GTG GGC TTT TGA CAG GT | 106 | 87-107 | L1 gene |
| Reverse | 6R | GGT TAT CGC CTC CCC CAA ATG G | | 171-193 | |

**PCR Kit: Roche Faststart SYBR Green mastermix**

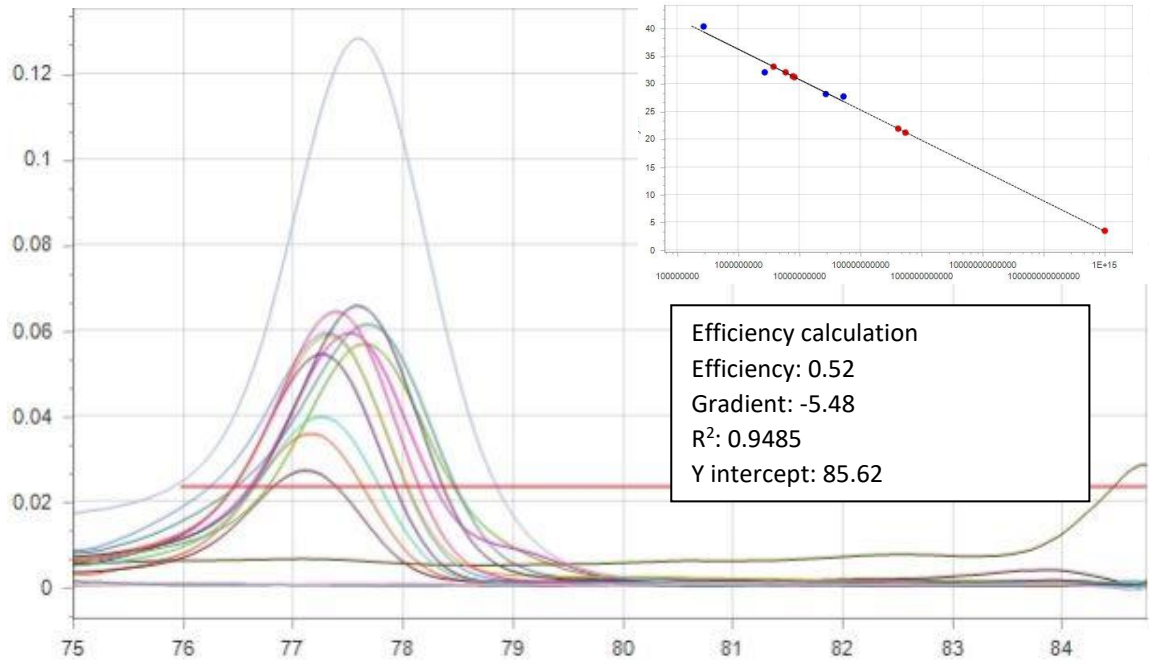
| **Reagent mix** | **Volume (20µL)** |
|---|---|
| Sterile distilled water | 8µL |
| 2x SYBR green mastermix | 10µL |
| 10 µM 6F87 primer | 0.5µL |
| 10µM 6R171 primer | 0.5µL |
| DNA | 2µL |

| **PCR controls** | **Description** |
|---|---|
| Positive | HPV 6 positive sample |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** 6F/6R

| **Cycling Parameters** | **Temp (˚C)** | **Time** | **No. Cycles** |
|---|---|---|---|
| Hold | 95 | 10 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>57<br>72 | 15 sec<br>30 sec<br>30 sec | 45 |
| Melt | 75-85 (0.2 increments) | Three second hold | 1 |

**Standard curve and melt image**



# Appendix 7 - 6F106/6R240 qPCR SOP

| **6F106/6R240 qPCR** |
|---|

| **Nucleic acid extraction** | **Kit** |
|---|---|
| DNA | Qiagen DNeasy Blood and Tissue kit with (FFPE pre-treatment step if required). |

| **Primers** | **Name** | **Sequence (5'-3')** | **Size** | **Position** | **Target** |
|---|---|---|---|---|---|
| Forward | 6F | AGG TAA TGG CCT GTG ACT GC | 155 | 106-126 | L1 gene |
| Reverse | 6R | TGT CTG CTG AAG TAA TGG CCT | | 240-261 | |

**PCR Kit: Roche Faststart SYBR Green mastermix**

| **Reagent mix** | **Volume (20µL)** |
|---|---|
| Sterile distilled water | 8µL |
| 2x SYBR green mastermix | 10µL |
| 10 µM 6F106 primer | 0.5µL |
| 10µM 6R240 primer | 0.5µL |
| DNA | 2µL |

| **PCR controls** | **Description** |
|---|---|
| Positive | HPV 6 positive sample |
| Negative | Control/Blank DNA extraction |
| Non-template control | Nuclease free water |

**PCR programme name:** 6F/6R

| **Cycling Parameters** | **Temp (˚C)** | **Time** | **No. Cycles** |
|---|---|---|---|
| Hold | 95 | 10 min | 1 |
| Denature<br>Anneal<br>Extension | 95<br>57<br>72 | 15 sec<br>30 sec<br>30 sec | 45 |

| | | | |
|---|---|---|---|
| Melt | 75-85 (0.2 increments) | Three second hold | 1 |

**Melt image**