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Molecular diagnosis of high-risk Human Papilloma Virus infection in a South African patient cohort

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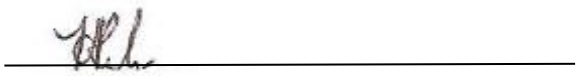
Supervisors: Dr L. Marcus MMed (Clin Path) and Dr P.M. Durand MMed (Clin Path), PhD

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Masters of Science in medicine.

Johannesburg, 2018

Declaration

I, Hendrik P. Earle, declare that this dissertation is my work. It is submitted for the degree of Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



9th day of October 2018.

Outcomes

I. Poster presentation 5th FIDSA Congress 2013, Champagne Sports Resort, Drakensberg, South Africa

Title:

mRNA Human Papilloma Virus E6/E7 integration and the correlation with high-risk HPV genotyping: some epidemiological findings.

Authors:

H.P. Earle

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List of abbreviations

ASCUS: Atypical squamous cells of undetermined significance

ATHENA: Addressing the need for advanced HPV diagnostics

BLAST: Basic local alignment search tool

bp: base pair

CIN: Cervical intraepithelial neoplasia

DDR: DNA damage response

DNA: Deoxyribonucleic acid

dNTP: deoxy-nucleotide triphosphate

DTS: direct tube sampling

E: Early region

EC/TZ: Endocervical transformation zone

HIV: Human immunodeficiency virus

HPV: Human papilloma virus

hrHPV: high-risk human papilloma virus

HSIL: High-grade squamous intraepithelial lesion

HSPG: Heparan sulfate proteoglycan

IARC: International Agency for Research on Cancer

ICC: Invasive cervical cancer

L: Late region

LB: Lysogeny broth

LBC: Liquid-based cytology

LCR: Long control region

LEEP: Loop electrosurgical excision procedure

LLETZ: Large loop excision of the transformation zone

lrHPV: Low-risk human papilloma virus

LGSIL: Low-grade squamous intraepithelial lesion

MCS: Multiple Cloning Site

mRNA: Messenger Ribonucleic acid

N/A: Not available

NCBI: National Centre for Biotechnology Information

NLS: Nuclear localisation signals

ORF: Open reading frame

PCR: Polymerase chain reaction

PFU: *Pyrococcus Furiosus*

PPV: Positive predictive value

pRb: Retinoblastoma protein

qHPV: Quadrivalent HPV

TOCE: Tagging oligonucleotide cleavage and extension

VIA: Visual inspection with acetic acid

VILI: Visual inspection with Lugol's iodine

VLP: Virus-like particle

List of measurement units

A: ampere

g: gram

g: relative centrifugal force (1.118×10^{-5})

h: hour

min: minute

mL: millilitre

mM: millimole

ng: nanogram

ng/µL: nanogram per microlitre

nm: nanometre

s: second

V/cm: volt per centimetre

µM: micromolar

µL: microlitre

U/µL: Units per microliter

Abstract

Molecular pathology laboratories use DNA and mRNA PCR assays to detect hrHPV infections that are associated with carcinomas. The objective of this study was to determine the prevalence of HPV sub-types in HPV E6/E7 mRNA positive samples from the study cohort. HPV DNA genotyping was used to determine HPV prevalence. Epidemiological studies are important for public health, because they focus on the patterns of disease occurrence and the factors that influence these patterns. A total of 160 LBC samples, sent for routine screening of HPV E6/E7 mRNA were used for this observational descriptive study. Demographic information indicated that the samples had been collected from Gauteng and Limpopo provinces. The ages of the patients varied from 18 years to 73 years and patients' ethnicities were seldom listed. HPV DNA genotyping results indicated that HPV 16 with a result of 18.83% was the most common HPV sub-type detected and this prevalence of HPV 16 was consistent with findings reported in the literature. HPV 59 was the second-most commonly HPV sub-type detected with a result of 13.64%. HPV 18 had a relatively lower prevalence result of 6.49%, together with HPV 51 and was ranked as the ninth commonest HPV sub-type. The 10 most prevalent HPV sub-types detected in this study were all from group 1A carcinogens, except for one, HPV 35, that was detected as 6 most commonest HPV sub-type with a percentage of 9.09 %. The results indicate that the new 9vHPV vaccine would be beneficial for South Africa because it vaccinate against 4 of the 5 most prevalent HPV sub-types detected in this study.

Chapter 1: Introduction

Certain human papilloma virus (HPV) sub-types known as the high-risk human papilloma virus (hrHPV) sub-types cause cervical cancer in women (1). Molecular pathology laboratories use deoxyribonucleic acid (DNA) and messenger ribonucleic acid (mRNA) polymerase chain reaction (PCR) assays to detect HPV infections.

The prevalence of different HPV sub-types varies among diverse populations (2). Various hrHPV genotypes have different oncogenic potentials (3). Therefore the prevalence of HPV sub-types in a population is relevant to the management of HPV infections and the prevention of cervical cancer (3).

In 2010 the South African HPV Advisory Board established three algorithms for screening cervical cancer (4). These guidelines were revised in 2017 by Botha *et al.* (5). The revised screening guidelines for cervical cancer include only two algorithms (5). The first algorithm, use cytology as primary screening method and the second algorithm use HPV testing as primary screening method (5).

Effective vaccination against HPV could prevent cervical cancer caused by hrHPV sub-types (6). Although a challenge of this strategy is that vaccines are preventative and decades are required to determine their effectiveness (6).

1.1 Introduction to HPV

Papilloma viruses are members of the *Papillomaviridae* family (7). HPV is a small, non-enveloped double-stranded DNA virus (7,8). Its genome is made up of eight genes and consists of 7900 base pair (bp) (8). The genome is divided into three regions: an early region (E), late region (L), and a long control region (LCR) (3,9). The early region of the genome encodes early genes E1, E2, E4, E5, E6, and E7, which are involved in viral replication, transcription regulation, and oncogenesis (3). The late region of the genome encodes structural proteins L1 and L2 (3). The icosahedral capsid of HPV is composed out of these two structural proteins (3). This capsid includes 72 capsomeres (3,8). Each capsomere is a pentamer of the major capsid protein, L1 (10). The minor capsid protein, L2, is present in much lower amounts than L1, with a maximum of 72 copies per virion at the vertices (11). The LCR region regulates the control of DNA replication and transcription of the eight open reading frames (ORFs) (3). The LCR region contains the viral early promoter and transcriptional enhancer, the viral origin of replication, the late polyadenylation site and the late regulatory element that controls late gene expression at various post-transcriptional levels (12). This region has the highest degree of variation in the HPV genome (3). Figure 1.1 shows the structure of HPV.

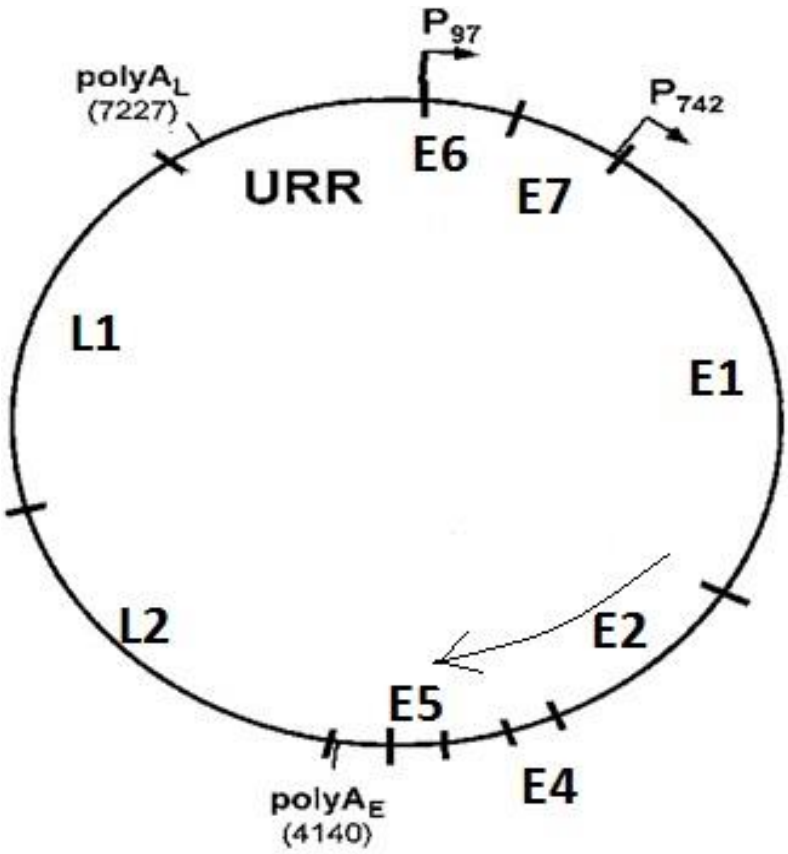


Figure 1.1: Structure of HPV. Figure adapted from Fehrmann *et al.* (13).

HPV comprises over 100 genotypes that are categorised according to their genomic sequence into five genera: alpha, beta, gamma, mu, and nu (3,14). Most HPV types cause benign warts of the skin or genital region (15). Alpha papilloma viruses are categorized as high-risk or low-risk according to their potential to cause cancer (15). In 2012, the International Agency for Research on Cancer (IARC) categorized HPVs into the following 3 groups: group 1 carcinogens, group 2A carcinogens and group 2B carcinogens (15). Group 1 carcinogens are carcinogenic to humans and this group include: HPV sub-types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (15). Group 2A carcinogens are classified as probably carcinogenic to humans and this group include HPV 68 (15). Group 2B carcinogens are possibly carcinogenic to humans and include: HPV sub-types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, and 97 (15). Group 1 carcinogens and group 2A carcinogens are responsible for 96% of cervical cancers (15).

New papilloma virus types are assigned when the full-length genome has been deposited at the International HPV Reference Center at the Karolinska Institutet, Stockholm, Sweden, and the L1 ORF sequence is verified as differing by more than 10% (less than 90% similarity) from the closest known HPV type (15). Differences between 2% and 10% homology define a sub-type and differences less than 2% define a variant (15).

1.2 Life cycle and pathogenesis of HPV

Papilloma viruses are host-specific and successful infection depends on epithelial differentiation (14,16). HPV infects the basal layer of the epidermis, and its genome replicates in synchrony with cellular DNA replication (13). Infection of the squamous epithelial cells of the genitalia and anal verge occurs through the interaction of the virus with the host cellular receptors (17). HPV uses the L1 proteins to bind to the cell membrane (10). HPV binds to cellular receptors that are trypsin-sensitive structures (10,18). This receptor can either be a protein (usually a glycoprotein) or a carbohydrate structure localised on glycoproteins or glycolipids (19).

The linear polysaccharide Heparan sulfate proteoglycan (HSPG) is proposed as the initial binding receptor for HPV (10,20). HPV binds to HSPG on the extracellular matrix and the cell membrane (20). This causes proteolytic cleavage of the capsid, leading to conformational changes that expose a Furin cleavage site on the L2 proteins (14,20). Cleavage of the L2 proteins is caused by the pro-convertase enzyme Furin, leading to the elimination of one of the putative nuclear localisation signals (NLSs) (14). When papilloma viruses are exposed to Furin inhibitors, they have a lower infectivity potential (14). After HPV binds to the cell surface receptors it enters the cell through either clathrin-mediated or caveolin-mediated endocytosis (21). When the virus enters the host's epithelial cells, its coat is dismantled so that the virus's genome gains access to the transcription and replication machinery of the infected cells (20). HPV does not

encode DNA polymerase activity for viral genome replication and therefore the host DNA replication machinery is required (22).

The infected cells enter the suprabasal layer of the epidermis, where the cells differentiate from the stratum spinosum to the stratum corneum (13). HPV genes are expressed as autonomous replicating episomal or extra-chromosomal elements, or as integrated DNA for the E6 and E7 genes (17,23).

After leaving the basal membrane, the infected cells initiate the differentiation program (22). DNA replication is down regulated in the differentiated cells that exit from the cell division cycle (22). Therefore HPV needs to reactivate cell division among the differentiation initiated cells (22). Proteins produced by the transcription of the E6 and E7 genes bind to tumour suppressor proteins such as p53 and the retinoblastoma protein (pRb) (24). The p53 protein is a tumour suppressor protein; its functions are to induce cell cycle arrest, DNA repair, senescence, and apoptosis (25). The pRb protein is responsible for the major G1 checkpoint (restriction point) blocking S-phase entry and cell growth, and for promoting terminal differentiation by inducing both cell-cycle exit and tissue-specific gene expression (26).

E6 and E7 are important in maintaining infected cells in an undifferentiated state, but terminal differentiation is required for the productive replication of HPV (22). The inhibition of E6/E7 expression by E2 might promote cellular differentiation, which is suitable for the viral productive lifecycle (22). E2 has transcriptional transactivator activity, as well as the capacity to bind to the viral DNA replication factor E1 (22). E1 has DNA helicase and ATPase activities and weak DNA-binding capacity (22). Through its interaction with E2, E1 binds to the replication origin, which is essential for the initiation of viral DNA replication (22). Integration of the E6

and E7 genes disrupts the E2 gene (27). E1 proteins will keep the cell in the S-phase of cell division and it will trigger the activation of a DNA damage response (DDR) and ATM pathway (22). The activation of DDR might facilitate HPV DNA replication (22).

Viral gene products E4 and E5 regulate late viral functions, playing a role in virion release and immune evasion, respectively, and are also required for productive viral replication (16). The alpha papilloma virus species and all the carcinogenic types were derived from a common ancestor E5 protein code, whereas other HPVs either lack a definable E5 ORF or a translation start codon for E5 (28). The HPV E5 ORF is classified into four groups – alpha, beta, gamma, and delta – which correlate with different clinical manifestations, notably oncogenic potential (28). Variants of this protein appear to increase the likelihood of oncogenic transformation following persistent infections (28). The absence of E5 indicates that the protein is not essential for the life cycle of these viruses but rather enhances infection and transformation (28). The lack of an E5 ORF represents a factor that hampers the fully replicative cycle of these viruses (28). Some beta HPVs are detected together with HPV 3 or related genotypes, and such co-detection with E5-encoding HPV suggests that beta HPVs benefit from E5 delivered by the co-infecting HPV sub-type (28).

Following the genome amplification, in the terminally differentiated cells, the synthesis of capsid proteins are triggered (22). A late promoter activates the capsid genes L1 and L2 (12). The capsid proteins assemble into virions that encapsidate viral genomic DNA (22). The progenitor virions are released externally with keratinocytes that are shed (22). Figure 1.2 shows abnormal epithelial differentiation induced by HPV. The arrows indicate the direction of epithelial differentiation, from the stratum basale to the stratum corneum.

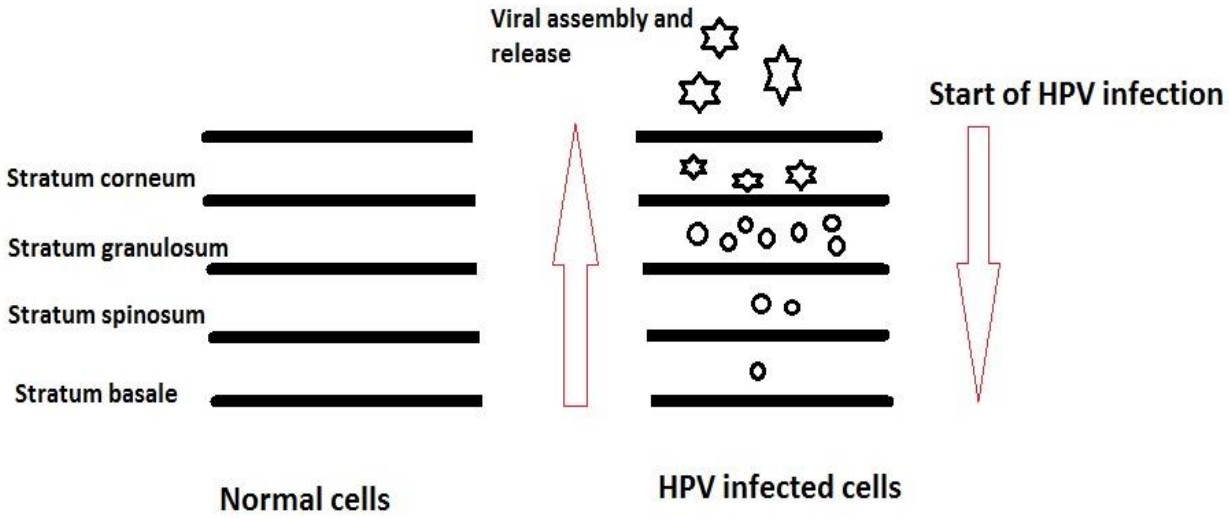


Figure 1.2: Schematic representation of abnormal epithelial differentiation induced by HPV. Figure adapted from Fehrmann *et al.* (13).

1.3 Evolution of HPV

Literature indicates that HPV evolved through two processes, namely convergent evolution and co-evolution. HPV demonstrates convergent evolution because the high- and low-risk types of HPV have independently evolved twice in the alpha genus (29). This style of evolution relates to a phenotypic strategy that uses ecological opportunities within human populations (29). An example is virulence versus per-contact transmissibility (29). High-virion production enhances transmissibility but also provokes an immune response which leads to clearance and limited persistence (29). Conversely, low-virion production increases persistence at the cost of diminished transmission probability per sexual contact (29). The low risk HPV (lrHPV) types use the former strategy, whereas hrHPV types use the second strategy (29).

The observation that papilloma viruses cause benign infections and are unable to cross the hosts' species-barrier has led to the hypothesis that HPV evolved through host-linked evolution (30,31). The co-evolution theory for papilloma viruses has been criticised. The orthogenetic definition of co-evolution states that parasites of closely related host species should be closely related

themselves, and should cluster together in the parasite phylogenetic tree (30). Papilloma viruses display discrepancies between both trees, and the parasites and hosts might not have co-evolved (30). With an increase in the number of papilloma virus sequences – and their associated hosts, it is clear that papilloma viruses and their hosts did not follow identical evolutionary paths (30).

Several discrepancies that do not support co-evolution can be observed in the phylogenetic tree (30). For example, as mentioned earlier, HPVs are classified into five genera (alpha, beta, gamma, mu, and nu) which are dispersed throughout the phylogenetic tree (30). Strict co-evolution would place the branch leading to the nonhuman-primate papilloma viruses basal to HPVs, rather than intermingled as observed (30). Evolutionary events such as cross-species infection, recombination, and virus duplication have been suggested to explain the observed conflicts (30). As there are no cross-species infections for *Papillomaviridae*, it is unlikely that horizontal gene transfer played a role in the evolution of the *Papillomaviridae* (30). However, a study that investigated the influence of horizontal gene transfer identified one potential cross-species transmission event, involving ancestors of a porcupine virus (EdPV1) and HPV 41 (30).

In the early 1960s, another theory of co-evolution was proposed (30). According to this more recent theory, the evolution of parasites follows the evolution of host resources rather than the evolution of the host species per se (30). This theory explains the shape of the *Papillomaviridae* phylogenetic tree, because specific events in the evolution of the host created new ecological niches to which papilloma viruses could adapt (30). Therefore, the availability of new niches to infect would have driven the evolution of papilloma viruses (30). Niche sorting and virus–host linked speciation were key determinants in the evolutionary history of papilloma viruses (30).

1.4 HPV epidemiology

Epidemiological studies are important for public health. Epidemiology focuses on the patterns of disease occurrence and factors that influence those patterns. However, the diversity of HPV makes it difficult to form a precise epidemiologic definition of which type of virus is responsible for which disease (32) because different HPV types are more prevalent in different cancers.

HPV is the most common sexually transmitted infection in the world (33). Women who are sexually active face a lifetime risk of 80% for contracting an HPV infection (33). The development of cervical neoplasia into cervical cancer usually takes a decade or more, hence women older than 30 years are most at risk for cervical cancer (34). The numbers of women infected with HPV are higher in developing countries than developed countries (35). It is estimated that 493 000 new cervical cancer cases and 274 000 related deaths occurred globally in 2002, with more than 80% of those cases having occurred in developing countries (36,37). More recent data indicated that there were 528 000 new cervical cancer cases and 266 000 deaths worldwide in 2012 (38). South Africa has an annual incidence of 7735 cervical cancer cases, with an estimated annual death rate of 4248 related cases (39).

A study of a population in Tshwane District of Gauteng province showed that the most common HPV infections among this population were HPV types 16, 51, 58, 45, 35, 18, 33, and 52, as well as two possibly (group 2B) high-risk types, namely 53 and 66 (40). In another study, five clinics in Tshwane District that were known not to offer cervical cancer screening services were selected to participate. The study examined the type- and age-specific prevalence of HPV infections and cytological abnormalities among women (41). The results indicated that women younger than 40 years of age had the highest infection rate of HPV as well as multiple HPV sub-type infections. These findings could be explained by the relatively high number of sexually

active women who were younger than 40. Women older than 30 years showed the highest degree of cervical neoplasia, with a decline after 44 years of age.

At least 21% of women in South Africa harbour a cervical HPV infection at any time (33). The prevalence of hrHPV types might be as high as 60% in certain populations and rises to 85% among women who are also infected with human immunodeficiency virus (HIV) (34).

Bzhalava 2013 *et al.* (32) conducted a systematic review of the prevalence of mucosal and cutaneous HPV sub-types. Publications from January 1990 to November 2011 were selected from Medline. Bzhalava 2013 *et al*. (32) stated that HPV prevalence increased with the severity of cervical disease, from 12.6% in normal cytology to 89.5% in cases of invasive cervical cancer (ICC). They also noted that HPV type 16 was the most frequently detected sub-type in every cytological grade. The results showed that HPV types 16, 18, and 45 were more often detected in ICC cases than the other hrHPV types, which were more often detected in intermediate cervical diagnosis (cervical dysplasia < cervical intraepithelial neoplasia (CIN) 3).

During 2012 McDonald *et al.* (42) studied the prevalence of hrHPV genotypes among HIV-negative women in Cape Town, South Africa. In that study, 20.7% of women were hrHPV positive, and women with CIN had the highest positive rates. The HPV prevalence decreased with increasing age among women without CIN. However, a bimodal age curve was observed among women with CIN. HPV 16 and 35 were the most common hrHPV genotypes in all ages and CIN groups. HPV 45 became more frequent among older women with CIN grade 2 or 3 compared with other HPV sub-types. Younger women (17–29 years of age) had more multiple hrHPV genotypes overall, and in each cervical disease group, than older women (40–65 years of

age). In conclusion, McDonald *et al.* (42) indicated that in their study HPV types 16, 35, and 45 were the most frequently detected among patients with CIN 2/3 lesions.

In 2012, Liverani *et al.* (27) combined HPV DNA genotyping with HPV E6/E7 mRNA detection to evaluate the prevalence of HPV genotypes in women diagnosed with CIN grade 2 or higher. The results showed that HPV 16 was the most prevalent genotype found in histologically confirmed high-grade cervical lesions, and HPV 31 was the second most prevalent type. Among the 979 women who had lesions of CIN grade 2 or lower, 588 (60.1%) tested positive for hrHPV DNA types, and 98 (10.1%) tested positive for HPV E6/E7 mRNA from HPV types 16, 18, 31, 33, or 45.

HPV types 16 and 18 are the most prevalent hrHPV sub-types in the world and are also the most oncogenic (35,43-46). HPV types 16 and 18 are present in more than 50% of high-grade squamous intraepithelial lesions and in 80% to 90% of cervical cancers (47). In Africa, HPV 35 is as common as HPV 16 is in the rest of the world, followed by HPV sub-types 31, 45, 56, and 58 (43). In general, HPV infections are high in sub-Saharan Africa, with ICC prevalence (31.7 per 100 000) and mortality (22.9 per 100 000) rates that are among the highest in the world (42). However, the proportional prevalence of hrHPV-type infections caused by HPV 16 is lower in sub-Saharan Africa than in the rest of the world (42). The low proportional infection rate of HPV 16 in the region indicates that the current vaccines would not have the same effect in sub-Saharan Africa as in the rest of the world (42).

The difference in HPV prevalence among different populations across the globe seems to support the more recent theory of co-evolution. As mentioned earlier, this theory states that the evolution of parasites follows the evolution of host resources rather than the evolution of host species (30).

The theory might explain why certain HPV variants became more prevalent in some populations as *Homo sapiens* migrated out of Africa.

1.5 Cervical cancer screening

Cervical cancer screening strategies should ideally detect cancer precursor lesions that will progress to invasive cancer (48). Most HPV infections, including CIN1 and CIN2 cases, are transient and will not lead to CIN3 or ICC (48). A focus on the detection of transient infections causes unnecessary psychological distress and physical discomfort for the patient, because of the diagnostic and treatment procedures; these issues may increase the risk of complications in future pregnancies (49).

The addressing the need for advanced HPV diagnostics (ATHENA) study (50) evaluated the Cobas HPV assay as a primary screening test for cervical cancer in women aged 25 years or older. During the study, 42 209 women (aged ≥25 years) were enrolled and received cytology and hrHPV testing. The conclusions were that HPV primary screening was as effective as a hybrid screening strategy that used cytology (if patients were 25–29 years old) and co-testing (for patients 30 years or older).

According to the South African HPV advisory board, all South African women should start cervical cancer screening at 25 years of age or at the time of diagnosis of HIV seropositivity (5). Variation exists in the sophistication of health systems in different geographical areas of South Africa (5). Guidelines for cervical cancer screening in South Africa include two algorithms and these two algorithms can be divided into 4 components: primary screening, secondary/triage screening, treatment and follow-up testing. These components will be discussed in the following section.

1.5.1 Primary screening

Even though the South African HPV advisory board prefer HPV testing to cytology, cervical cytology and HPV testing are both considered suitable for screening cervical cancer in South Africa and screening practitioners or facilities should choose the most appropriate test for their setting (5).

1.5.1.1 Cervical cytology screening

Cervical cytology screening intervals can vary from every 5 years for HIV unknown or HIV negative women to every 3 years for HIV positive women in low resource settings (5). Cervical cytology screening intervals can vary from every 3 years for HIV unknown or HIV negative women to yearly for HIV positive women in high resource settings (5). Cervical cytology results that are negative for intra-epithelial lesions or malignancy are low risk for the development of cervical cancer and screening can continue (5). Cervical cytology results indicative of atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LGSIL) are medium risk for the development of cervical cancer and triage testing is recommended (5). Follow-up testing is recommended for negative triage test results (5). Treatment should start immediately after positive triage test results (5). High-grade squamous intraepithelial lesion (HGSIL) cervical cytology results should be treated (5). After treatment, follow-up testing is necessary (5). Figure 1.3 is a summary of the guidelines for primary cytology screening in South Africa.

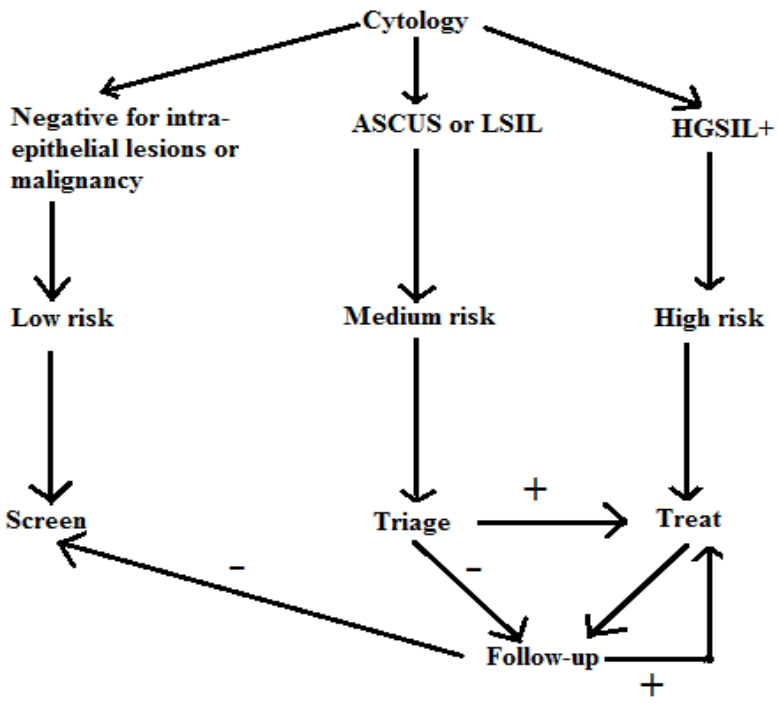


Figure 1.3: Guidelines for primary cytology screening in South Africa. Figure adapted from Botha *et al.* (5).

1.5.1.2 HPV testing

HPV screening intervals can vary from every 10 years for HIV unknown or HIV negative women to every 5 years for HIV positive women in low resource settings (5). HPV screening intervals can vary from every 5 years for HIV unknown or HIV negative women to every 3 years for HIV positive women in high resource settings (5). hrHPV negative screening results are low risk for cervical dysplasia and screening can continue (5). Non-discriminatory tests that are positive for hrHPV sub-types and discriminatory tests that are positive for other hrHPV sub-types are medium risk for cervical dysplasia and triage testing is recommended (5). Follow-up testing is recommended if the triage test results are negative (5). Treatment is recommended if triage test results are positive (5). Positive discriminatory tests that are positive for highest risk HPV sub-types in HIV negative women younger than 35 years of age are medium risk for cervical dysplasia and triage testing is recommended (5). Follow-up testing is required if the triage test results are negative (5). Discriminatory tests that are positive for the highest risk HPV

sub-types for women older than 35 years of age or women that have been diagnosed with HIV are high risk for cervical dysplasia and treatment is required (5). After treatment follow-up testing is necessary (5). Figure 1.4 is a summary of the guidelines for primary HPV screening in South Africa.

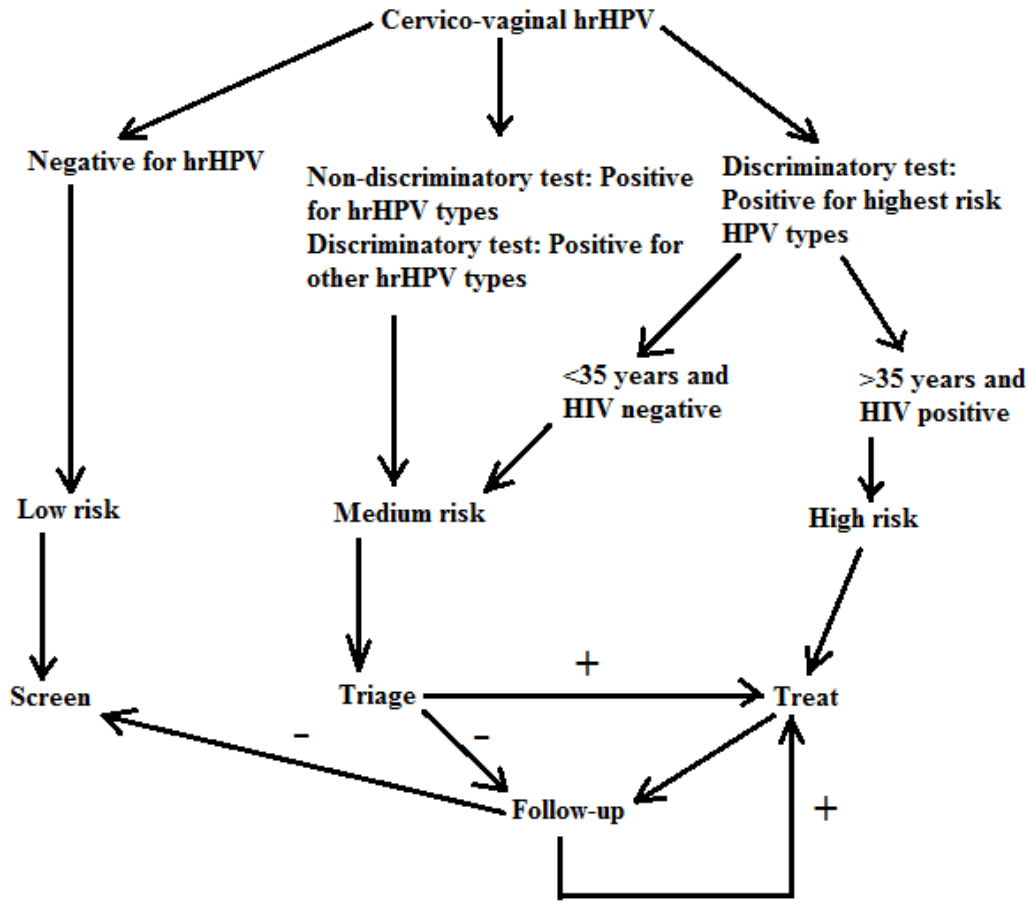


Figure 1.4: Guidelines for primary HPV screening in South Africa. Figure adapted from Botha *et al.* (5).

1.5.2 Secondary/triage testing

Triage testing will avoid over treatment of women who do not have a high risk for severe dysplasia (5). Triage testing include cytology, HPV testing, immunocytochemistry or simple visual inspection with colourant (5). Triage is usually with a different test to the initial screening test (5). Visual inspection with acetic acid (VIA) or visual inspection with Lugol's iodine (VILI)

can be used in low resource settings and if cytology or HPV testing are indicative of medium risk results (5). Conventional cytology or liquid-based cytology (LBC) can be used in low or high resource settings when the cytology or HPV triage test results were medium risk for cervical dysplasia (5). Cyto-staining or similar biomarkers can be used in high resource settings when cytology or HPV triage test results are medium risk for cervical dysplasia (5). HPV testing with or without partial genotyping can be used in high resource settings if the cytology result is indicative of medium risk for cervical dysplasia (5).

1.5.3 Treatment of cervical neoplasia

The 2006 Consensus Guidelines published by the American Society for Colposcopy and Cervical Pathology and its partner organisations utilise a two-tiered system, in which histologically diagnosed CIN1 is classified as a low-grade lesion and CIN2 or CIN3 are classified as high-grade precursors (49). If a histological diagnosis is indicative of CIN1, no treatment is recommended because most low-grade lesions regress spontaneously (49).

The risk of CIN2 progressing to invasive cancer is low, but CIN2 is used as the treatment threshold for safety reasons. That is, the diagnosis of CIN2 has limited replicability and validity because CIN2 regresses spontaneously in most cases (49). Surgical treatment options for CIN2/3 include ablative methods that destroy the affected cervical tissue *in vivo* (51). These methods include cryotherapy, laser ablation, electro fulguration, and cold coagulation, as well as excisional methods that remove the affected tissue and provide a specimen for pathological examination (51). The excisional methods include cold-knife conization, laser conization, electrosurgical needle conization, and loop electrosurgical excision procedures (LEEP) (49). LEEP is no more effective than other methods but it has clinical advantages – such as reduced operating time and lower rates of haemorrhage, pain and infection (49). In addition, LEEP is less

expensive and technically simpler than laser treatment, which makes it the preferred method of treatment (49).

Atypical glandular cell (AGC) cytology results needs adequate investigation of the endocervical canal and endometrium (5). The South African HPV advisory board recommended that woman younger than 30 years of age with a single AGC result may be treated with antibiotics and the cytology must be repeated (5). Women older than 30 years of age with AGC or where a repeat cytology remains abnormal, an endocervical or large loop excision of the transformation zone (LLETZ) sample and endometrial sample must be obtained for cytology and/or histology (5).

The South African HPV advisory board prefer excisional treatment with a loop procedure treatment option for abnormal screening test results (5). The cervical surface must be stained with iodine before the procedure to identify and include all abnormal epithelium (5). Histology of the excised specimen is preferred (5). In cases where infiltrating cancer has been excluded, the transformation zone is visible and resources for histology are limited, ablative therapy with cryo-treatment or cauterisation is acceptable (5). The management of normal and abnormal triage results recommended by the South African HPV advisory board will be discussed in the next section.

1.5.3.1 VIA or VILI triage test

Biopsy is necessary for VIA or VILI results indicative of a possible invasive lesion (5). Treatment is not required for VIA or VILI results indicative of no lesions (5). Small lesions observed with VIA or VILI should be treated with cryotreatment or LLETZ (5). LLETZ should be used for the management of large lesions observed with VIA or VILI (5).

1.5.3.2 Cytology triage test

No treatment is required for a normal cytology triage test results (5). Cytology results indicative of ASCUS or worse should be managed with cryotreatment or LLETZ (5). No treatment is required for normal cyto-stain or similar biomarker results (5). Positive cyto-stain or similar biomarker results should be managed with colposcopy or biopsy (5).

1.5.3.3 HPV triage test

No treatment is required for a normal HPV triage test results (5). Positive HPV triage test results should be managed with colposcopy and biopsy (5).

1.5.4 Follow-up after treatment

The South African HPV advisory board recommend that follow-up visits should be at shorter intervals than screening visits until all tests are normal (5). In low resource settings follow-up testing after treatment of woman that are HIV negative or younger than 35 years of age should be done every 5 years or until normal follow-up test results (5). In high resource settings follow-up testing after treatment of women that are HIV negative or younger than 35 years of age should be done every year or until normal follow-up test results (5). Women that are HIV positive or older than 35 years of age should have follow-up tests every year until follow-up tests are normal (5). In the case where follow-up tests are normal women should continue with screening (5). If a second abnormal test is observed, women should start retreatment (5). In low resource settings women can exit screening at 55 years of age or when they have had a hysterectomy (5). In high resource settings women can exit screening at 65 years of age or when they have had a hysterectomy (5). It is recommended by the South African HPV advisory board that HIV positive woman should never exit cervical cancer screening (5).

1.6 HPV prevention

Currently, three prophylactic HPV vaccines are licenced (52). The first vaccine is Gardasil, a quadrivalent HPV (qHPV) vaccine that vaccinate against HPV sub-types 16, 18, 6 and 11 (53). The second vaccine is Cervarix, a bivalent HPV vaccine that vaccinate against HPV sub-types 16 and 18 (53). The third and latest prophylactic vaccine is Gardasil®9, a 9-valent vaccine that vaccinate against HPV sub-types 6, 11, 16, 18, 31, 33, 45, 52 and 58 (53). These vaccines contain recombinant virus-like particles (VLPs) assembled from the L1 major capsid proteins of different HPV sub-types (53). The quadrivalent Gardasil and bivalent Cervarix vaccines are the only vaccines available in South Africa (54). The 9-valent HPV (vHPV) vaccine have been licenced in the US, Europe, Canada, Australia, Chile, Hong-Kong, Ecuador, South Korea, and New Zealand (53). The 9vHPV vaccine replaced the qHPV vaccine in the US (53).

Therapeutic HPV vaccines would be extremely valuable because they would eliminate already persistent HPV infections (36). The limiting factor for the development of therapeutic vaccines is that immunological determinants for viral persistence or regression are poorly defined (36). Hosts who have impaired cellular immunity face an increased risk of persistent HPV infection and carcinogenic progression (36).

Bharadwaj *et al.* (36) stated that ideally HPV vaccines should be chimeric – that is, prophylactic as well as therapeutic. This would lead to both humoral and cell-mediated immunity, so that new infections could be prevented while established HPV infections are eliminated (36). Chimeric VLPs – namely L2E7E6 fusion protein vaccine – in trial studies have been shown to enhance immunity to HPV 16 and also enhanced E6- and E7-specific T-cell immunity in healthy volunteers (43). Chimeric vaccines would be highly beneficial in populations that do not receive routine screening and already have HPV-related cervical disease (36).

1.7 Introduction to the methodology

As mentioned, epidemiological studies are important for public health; such studies focus on patterns of disease and the factors that influence those patterns. HPV epidemiological studies on mRNA positive samples indicate the clinically relevant HPV types in a population, because HPV mRNA is indicative of active HPV infections. In this study, HPV DNA genotyping was used to determine the HPV sub-types present in mRNA positive samples. Twelve samples that were representative of the 12 oncogenic HPV sub-types (group 1) were sent for sequencing to identify the variants present in these samples.

Chapter 2: Materials and methods

The study objective was to investigate the prevalence of HPV genotypes present in HPV mRNA positive samples. An Ethics clearance certificate (Appendix E) was obtained from the Human Research Ethics Committee (Medical). Samples sent for routine screening of HPV E6/E7 mRNA were collected from the Vermaak and Pathcare molecular laboratory in Centurion, Pretoria. All the samples that tested positive for HPV E6/E7 mRNA, between January 2013 and January 2015, were collected. A total of 160 LBC samples were collected. Samples collected from the Vermaak and Pathcare molecular laboratory included a total of 10 samples that were sent from the National Health Laboratory Service at Steve Biko Academic Hospital, for routine screening of HPV E6/E7 mRNA.

Demographic information indicated that the samples had been collected from northern Pretoria (22.62%), eastern Pretoria (11.88%), southern Pretoria (15.63%), western Pretoria (6.25%), Pretoria central (5.63%), and from nearby suburbs, towns and cities (37.99%). The latter category included Roodepoort, Johannesburg, Randburg, and Bela-Bela in Limpopo province. The ages of the patients varied from 18 years to 73 years, with an average of 31 years and 8 months. The patients' ethnicities were seldom listed, with roughly 15% of the samples being marked as from black African, white, coloured, or Indian and Asian patients.

For each LBC sample received, 1000 µL was transferred into an Aptima transfer tube which contained a buffer that stabilises RNA. Before the samples were transferred into the Aptima tubes they were stored at 25 °C, and after transfer they were stored at 8 °C. The Aptima HPV E6/E7 mRNA assay from Hologic® (USA) is a qualitative method for detecting mRNA from all the HPV sub-types in group 1 (HPV sub-types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), group 2A (HPV 66) and 1 HPV sub-type from group 2B (HPV 68) using a target capture method

(55). Capture oligomers bind to the HPV mRNA, which is then amplified by a transcription-based method (55). Detection of the integration of HPV into the host genome relies on detecting HPV E6/E7 mRNA with the direct tube sampling (DTS) system or the Panther from Hologic® (USA). Routine processing of samples send to the molecular laboratory of Vermaak and Pathcare included 3 negative calibrators and 3 positive calibrators as quality control. Figure 2.1 indicate the basic flow of the experimental protocol.

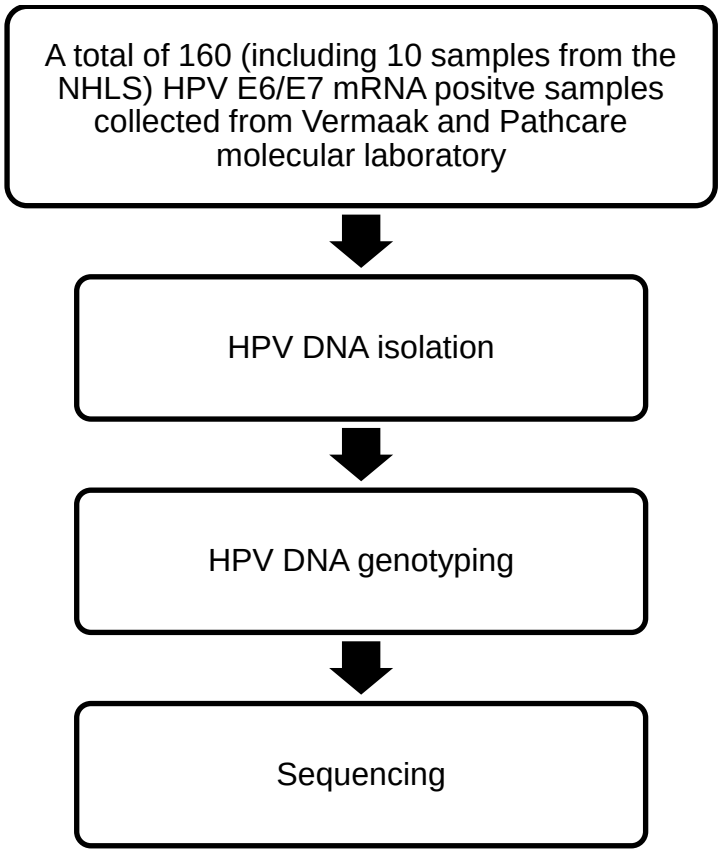


Figure 2.1: Basic experimental protocol.

2.1 HPV DNA extraction

HPV DNA was isolated from HPV E6/E7 mRNA positive samples, using the Roche High Pure viral nucleic acid isolation kit (Switzerland) and the ZR Viral DNA/RNA Kit™ from Zymo Research (USA).

2.1.1 Roche High Pure viral nucleic acid isolation kit

The working solution of the High Pure viral nucleic acid kit was made up as follows: Proteinase K was dissolved in 5 mL elution buffer, and the poly(A) carrier RNA was dissolved in 0.5 mL elution buffer and stored as 50 µL aliquots at -15 °C to -25 °C. Thereafter, 20 mL absolute ethanol was added to the inhibitor removal buffer and 40 mL absolute ethanol was added to each wash buffer.

After the working solutions had been prepared, the samples were isolated as follow: in a 1.5-mL RNase/DNase-free tube, 200 µL binding buffer supplemented with poly(A) and 50 µL Proteinase K was added to 200 µL of each sample. The samples were incubated at 72 °C for 10 min. After incubation of the samples, 100 µL binding buffer was added to each sample. A High Pure filter tube was combined with a collection tube for each sample, and the mixture was transferred to the upper reservoir. It was centrifuged at 8000 g for 1 min.

After the samples had been centrifuged, the flow-through in the collection tubes was discarded and the filter tubes were placed into a new collection tube. Then 500 µL inhibitor removal buffer was added to the samples and the samples were centrifuged at 8000 g for 1 min. The flow-through was discarded, and the filter tubes were placed into new collection tubes, after which 450 µL wash buffer was added to each sample and the mixture was centrifuged at 8000 g for 1 min. The flow-through was discarded, and the filter tubes were placed into new collection tubes. Again 450 µL wash buffer was added to each sample and centrifuged at 8000 g for 1 min. The samples were centrifuged at maximum speed (13000 g) for 10 s, after which the flow-through was discarded and the filter tubes were placed into new collection tubes. Then 50 µL elution buffer was added to each sample and the mix was centrifuged at 8000 g for 1 min. The flow-through was transferred into 1.5 mL RNase/DNase-free tubes.

2.1.2 ZR Viral DNA/RNA Kit™

The ZR Viral DNA/RNA Kit™ features a single buffer system, which facilitates complete viral particle lysis and allows for subsequent DNA/RNA binding to the matrix of the Zymo-Spin™ IC XL Column. In this study, HPV DNA was isolated as follows: 300 µL of lysis buffer was added to 100 µL of each sample. The samples were transferred to a Zymo-Spin™ IC XL Column in a collection tube and centrifuged at 12000 g for 1 min. The collection tube with the flow-through was discarded, and the column was placed into a new collection tube. Then 400 µL of DNA/RNA Prep buffer was added to the column and the mixture was centrifuged at 12000 g for 30 s. The flow-through was discarded and the column was placed into a new collection tube. Thereafter, 700 µL of DNA/RNA wash buffer was added to the column and centrifuged at 12000 g for 15 s. The flow-through was discarded, and the column was placed into a new collection tube.

The previous step was repeated with 400 µL DNA/RNA wash buffer. The flow-through was discarded, and the column was placed into a new collection tube. The column in the empty collection tube was centrifuged at maximum speed for 5 min to ensure complete removal of the DNA/RNA wash buffer. The column was placed into a DNase/RNase-free tube and 50 µL DNase/RNase-free water was added to the column. The sample was incubated for 1 min at room temperature and the mixture was then centrifuged at maximum speed for 30 s to elute the DNA.

2.2 HPV DNA Genotyping

HPV DNA genotyping was performed using the Anyplex™ II HPV28 DNA genotyping kit from Seegene® (South Korea), with the CFX96 Real-time Thermocycler from Biorad (USA). This stage of the experiment was aimed at determining the epidemiology of hrHPV sub-types in mRNA-positive samples for the sample population in Gauteng and Limpopo, South Africa.

The Anyplex™ II HPV28 detection kit is a qualitative *in vitro* test for detecting 28 HPV sub-types in LBC specimens and cervical swabs (55). According to the classification of HPV into high and low risk types by the IARC as described by Burd *et al.* (15), the Anyplex™ II HPV28 detection kit detects all 12 types in group 1 (HPV sub-types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), group 2A (HPV 68) and 6 of group 2B (HPV sub-types 26, 53, 66, 69, 73, and 82). The Anyplex™ II HPV28 also detects 9 lrHPV sub-types (HPV sub-types 6, 11, 40, 42, 43, 44, 54, 61 and 70). The target of detection for this assay is the L1 gene (56). This assay also detects a house keeping gene, human beta globin as an internal control (56). The Anyplex™ II HPV28 detection assay consists of two PCR reactions, set A and set B. Set A is a multiplex assay that permits the simultaneous amplification of target DNA from all 12 HPV sub-types in group 1, group 2A and one HPV sub-type from group 2B (HPV 66). Set B is a multiplex assay that permits the simultaneous amplification of target DNA of 5 HPV sub-types from group 2B and 9 lrHPV sub-types. The Anyplex™ II HPV28 detection assay represents Seegene's proprietary technologies and is based on a newly developed tagging oligonucleotide cleavage and extension (TOCE) technology, which enables detection of multiple pathogens in a single fluorescence channel on real-time PCR instruments (56). The TOCE technology uses artificial, template-based melting temperature instead of the current, probe-based melting temperature and distinguishes multiple targets in a single channel in a real-time PCR reaction (57).

The master mix for the real-time PCR reaction was prepared as indicated in Table 2.1. and Table 2.2. In both cases, 5 µL of the samples was added to the master mix and nuclease-free water was added to the final volume of 20 µL. The real-time PCR was performed using the CFX96 from Biorad®.

Table 2-1: HPV DNA genotyping master mix A

Reagent	Final concentration in 20 µL
HPV28 TOM A x4	x1
Anyplex PCR Master Mix (with UDG) x4	x1

Table 2-2: HPV DNA genotyping master mix B

Reagent	Final concentration in 20 µL
HPV28 TOM B x4	x1
Anyplex PCR Master Mix (with UDG) x4	x1

2.3 Sequencing

Sanger sequencing was used to sequence 12 samples representing the 12 oncogenic HPV sub-types (group 1) at Inqaba Biotech. Only HPV type 33 and 56 were successfully sequenced, and the sequencing results for HPV 33 were indicative of HPV 67. The other samples either had no amplification or showed mixed profiles. Because mixed profiles were observed with sequencing, cloning was performed to isolate single infections for sequencing. Before cloning, the quality of the DNA was tested on a 0.8% agarose gel with the following conditions: 10 V/cm and 3 A, for 1 h.

2.4 Cloning

HPV 33, 56 and 58 possessed sufficient genomic DNA concentrations to proceed with cloning. HPV 56 had already yielded successful sequencing results and it was thus included in the cloning experiment merely as a control. Cloning was performed with the InsTAclone™ PCR cloning kit from Thermo Fisher Scientific (USA). The InsTAclone™ PCR cloning kit takes advantage of the terminal transferase activity of Taq DNA polymerase and other non-proofreading thermostable

DNA polymerases. These enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favours direct cloning into a linearised cloning vector with single 3'-ddT overhangs. Recombinant clones are selected based on blue/white screening.

Blue/white screening is based on the following biochemical principle: β-galactosidase is a protein that is encoded by the *lacZ* gene of the *lac* operon. During cloning, a mutant form of this enzyme – called the ω-peptide – is used. This form of β-galactosidase has residues 11 to 41 deleted from its N-terminal. The mutant form of β-galactosidase is unable to produce a tetramer, which results in an inactive form of the enzyme. The mutated enzyme may become active when complemented with a α-peptide which contain the residues missing from the ω-peptide form of the enzyme. During cloning, the E. *coli* contains the ω-peptide form of the β-galactosidase enzyme, and the plasmid has the α-peptide. Cloning is based on the principle of the disruption of the complementation process between the two peptides.

The plasmid carries within the *lacZα* gene internal multiple cloning sites. The multiple cloning site (MCS) sequence can be cut by restriction enzymes so that a foreign DNA target can be inserted. Doing so would disrupt the production of the α-peptide. During the cloning process, the target DNA sequence is inserted into the MCS of the *lacZα* gene. This disrupts the production of the α-peptide, which leads to inactive β-galactosidase.

Active β-galactosidase can be detected by X-Gal solution. X-Gal is a colourless analog of lactose. It can be cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerises and oxidises to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. If no target DNA is inserted into the MCS of the *lacZα* gene, the α-peptide is produced. If the plasmid is transformed into the E. *coli*, the α-peptide and the ω-peptide

complement each other; this results in an active form of β-galactosidase, which will cleave lactose and result in the production of the blue colour. Figure 2.2 shows the map of the pTZ5/R/T cloning vector.

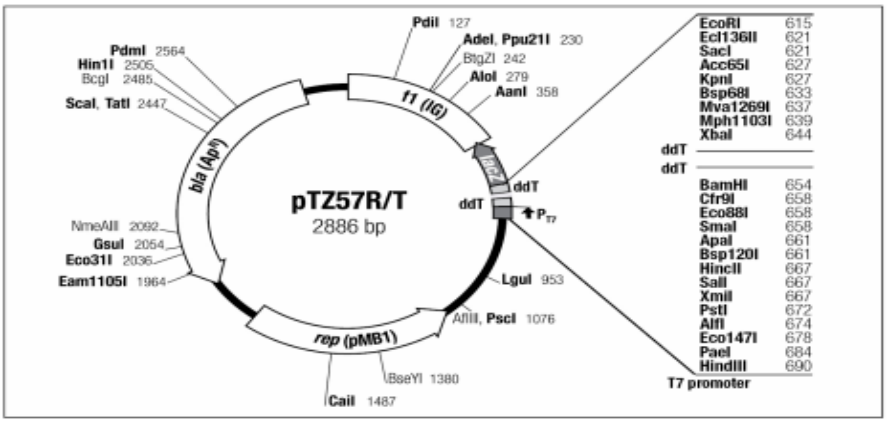


Figure 2-2: Map of the pTZ5/R/T cloning vector (figure reproduced from InsTAclone™ PCR cloning kit package insert).

2.4.1 Cloning PCR

Degenerate primers were used for cloning. The primer stock solution had a concentration of 100 µM. The published source of the sequences of the primers used for cloning was Sasagawa *et al.* (51), and the primers were synthesised by Inqaba Biotec. The sequences of the primers used are shown in Table 2.3.

Table 2-3: Primer sequences

Primer	Sequence
LCRF 1 (forward primer)	5'-WWARGGWGTRACCGAAAACGG-3'
LCRF 2 (forward primer)	5'-WWWGGGTCSAACCGAAAACGG-3'
LCRF 3 (forward primer)	5'-WRGGKTTAGGACCGAAAACGG-3'
E7R-2 (reverse Primer)	5'-TCWTCMTCHTCRTCTGAGCTGT-3'
E7R-3 (reverse primer)	5'-CACWAWATTKTGTGACGCTGTG-3'

A primer is degenerate when some of its positions have several base pairs (58). The symbols used in the degenerate primers to indicate multiple base-pair substitutes were as follows: R, W, Y, K, S, V, H, B, and N. The possible base pairs that are associated with the symbols are: R, A/G; W, A/T; Y, C/T; K, G/T; S, G/C; V, G/A/C; H, A/T/C; B, G/T/C; and N, A/G/C/T. The following reagents were used in the cloning PCR master mix:

- 10x PFU buffer, Thermo Scientific, USA
- 2.5 U/µL DNA polymerase from the hyper-thermophilic archaeum *Pyrococcus furiosus* (PFU)
- DNA polymerase enzyme, Thermo Scientific, USA
- 10 mM deoxynucleotide triphosphate (dNTP), Thermo Scientific, USA
- Water (H_2O), nuclease-free, Thermo Scientific, USA

Table 2.4 show the final concentrations of the reagents used in the master mix for the cloning PCR reaction. Finally, 5 µL DNA was added to the master mix and nuclease-free water was added to the volume of 25 µL.

Table 2-4: Cloning PCR master mix

Reagent	Final concentration in 25 µL
Primers 20 mM	2 mM
PFU x10 buffer	x1
PFU polymerase 2.5 U/µL	1 U/µL
dNTPs 10 mM	4 mM

The PCR cycling conditions were as follows. The mixture was denatured at 95 ºC for 5 min, followed by a continuous cycling step of 35 cycles. The 35 cycles included 95 ºC for 1 min, 55 ºC for 30 s, and 72 ºC for 1 min 30 s; the last step of the PCR was 72 ºC for 10 min. A 2%

agarose gel was set up with the conditions of 10 V/cm and 3 A for 1 h 45 min, to indicate if the PCR reactions had been successful. The reagents used for the gel were as follows:

- 3,6-anhydro-α-L-galacto-β-D-galactan (Agarose), Sigma-Aldrich, USA
- TRIS acetate-EDTA (TAE) buffer (0.4 M Tris acetate, 10 Mm EDTA, at pH 8.3), Sigma-Aldrich, USA
- Ethidium bromide 10 mg/mL, Thermo Scientific, USA
- MassRuler low-range DNA ladder, ready-to-use (60.8 ng/µL), Thermo Scientific, USA

After gel electrophoresis, the DNA was purified using the Macherey-Nagel (Germany) PCR clean-up gel extraction kit. The first step of the PCR clean-up was to cut the DNA band out of the gel and to measure the mass of the gel piece. In line with the manufacturer's instructions for a <2% agarose gel, 200 µL of buffer NTI was added for each 100 mg of gel. The gel was dissolved at 50 ºC for 5 to 10 min.

The samples were loaded into a NucleoSpin® gel and PCR clean-up column, which was inserted into a collection tube (2 ml). The samples were centrifuged at 11000 g for 30 s. The flow-through was discarded, and the column was placed back into the collection tube. Then 700 µL of buffer NT3 was added to the NucleoSpin® gel and PCR clean-up column and the mixture was centrifuged at 11000 g for 30 s. The flow-through was discarded, and the column was placed back into the collection tube. The samples were centrifuged at 11000 g for 1 min to remove buffer NT3 completely. The NucleoSpin® gel and PCR clean-up column was placed into a new 1.5 mL micro-centrifuge tube, and 15 µL to 20 µL buffer NE was added to the samples. The mixtures were incubated at room temperature (18–25 °C) for 1 min, after which the DNA was eluted by centrifuging the samples at 11000 g for 1 min.

2.4.2 DNA A-Tailing

During DNA A-tailing, Taq DNA polymerase adds adenine to the 3' end of the target DNA. This causes adenine of the target DNA to hybridise to the thymine 3' overhangs of the vector. After the cloning PCR, DNA A-tailing was performed. Reagents for the A-Tailing reaction included:

- 10x Taq buffer with $(NH_4)_2SO_4$ and 20 mM $MgCl_2$, which comprised 750 mM Tris-HCl (pH 8.8 at 25 ºC), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, and 20 mM $MgCl_2$ (Thermo Scientific, USA)
- 2 mM dATP, Thermo Scientific USA
- Taq DNA polymerase 5 U/µL, Thermo Scientific, USA
- 25 mM $MgCl_2$, Thermo Scientific, USA

The concentrations of the reagents in the master mix are listed in Table 2.5. Thereafter, 8 µL of the samples were added to the master mix. After the PCR products were added to the master mix, the mixtures were incubated at 70 ºC for between 15 min and 30 min. After DNA A-tailing, a PCR clean-up was performed using the Macherey-Nagel PCR clean-up gel extraction kit.

Table 2-5: DNA A-tailing master mix

Reagents	Final concentration in 15 µL
10x Taq buffer NH_4SO_4	x1
2 mM dATP	0.4 mM
Taq polymerase 5 U/µL	0.33 U/µL
25 mM $MgCl_2$	2.5 mM

The first step was to add NTI buffer to the samples at a ratio of 2:1 (buffer: original sample). The samples were then loaded into a NucleoSpin® gel and PCR clean-up column, which was inserted into a collection tube (2 mL). The samples were centrifuged at 11000 g for 30 s. The flow-through was discarded and the column was placed back into the collection tube, and 700 µL of

buffer NT3 was added to the NucleoSpin® gel and PCR clean-up column. The mixture was centrifuged at 11000 g for 30 s, and the flow-through was then discarded and the column was placed back into the collection tube. The samples were centrifuged at 11000 g for 1 min to remove buffer NT3 completely. The NucleoSpin® gel and PCR clean-up column was placed into a new 1.5 mL micro-centrifuge tube. Then 15 µL to 30 µL buffer NE was added to the samples and the mixture was incubated at room temperature (18–25 °C) for 1 min. The DNA was eluted by centrifuging the samples at 11000 g for 1 min. After the PCR clean-up of the DNA A-tailed samples, the samples were tested on a 2% agarose gel. The duration of the gel electrophoresis was 1 h 45 min under the following conditions: 10 V/cm and 3 A. After gel electrophoresis, the DNA was re-purified with the Macherey-Nagel PCR clean-up gel extraction kit as described previously.

2.4.3 Ligation

After PCR clean-up, the samples were ligated. The InsTAclone PCR cloning kit was used for the ligation reaction, and the ligation reaction master mix was prepared as shown in Table 2.6.

Table 2-6: Ligation reaction master mix

Reagent	Final concentration in 10 µL
Vector pTZ57R/T 55 ng/µL	11 ng/µL
5x Ligase buffer	x0.5
T4 DNA Ligase enzyme 5 U/µL	1 U/µL

A 2-µL insert (HPV DNA) was added to the master mix of the samples indicative of HPV sub-types 33 and 56 respectively. For the sample indicative of HPV 58, 1 µL of the insert was added to the master mix. Nuclease-free water was added to 10-µL volume. After the ligation reaction, the master mix was prepared, and the samples were then added and incubated at 22 ºC for 1 h.

2.4.4 Preparation of LB agar selection plates

Before the transformation process was started, the lysogeny broth (LB) agar plates were prepared. Reagents used for the LB agar selection plates included the following:

- Media for culture (LB plate and LB broth)
- Ampicillin, Sigma-Aldrich, USA
- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (X-Gal + dimethylsulfoxide), Sigma-Aldrich, USA
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) solution (IPTG + water), Sigma-Aldrich, USA
- Tryptone powder, Sigma-Aldrich, USA
- Sodium chloride
- Yeast extract EZMix™, Sigma-Aldrich, USA
- 3,6-anhydro-α-L-galacto-β-D-galactan (Agarose), Sigma-Aldrich, USA

The LB agar plates were prepared as follows: 2 g of tryptone, NaCl, 1 g yeast powder, and 3 g agar were dissolved in 200 mL water. After the reagents had been dissolved, 200 µL sterilised ampicillin stock was added. The solution was poured into a petri dish and incubated at room temperature. After the plate was set, 150 µL of sterilised X-GAL solution and IPTG solution were combined and 80 µL of this solution was streaked into the set plates.

2.4.5 Transformation reaction

Competent DH5α E. *coli* cells were prepared before cloning by being thawed for 15 to 20 min before the transformation reaction. After the cells were thawed, 10 µL of the ligated samples was added to the cells and the mixture was incubated on ice for 30 min. The samples were then incubated at 42 ºC for 90 s; thereafter, they were placed back on the ice immediately for 5 min.

Then 900 µL LB broth was added to each sample and the mixture was incubated at 37 ºC for 45 min. The samples were centrifuged at maximum speed for 5 min. After centrifugation, 900 µL supernatant was removed and the pellet was dissolved in the remaining fluid. The samples were plated onto agar plates and the mixture was spread all over the plates by streaking it in a clockwise direction. The plates were incubated overnight at 37 ºC. The next day, blue and white colonies were observed on the plates. The white colonies were picked off the plates, inoculated onto new plates, and incubated overnight at 37 ºC. The plates were incubated at 4 ºC until the colony PCR was performed.

2.4.6 Colony PCR

The reagents used for the colony PCR included the following:

- 100 µM forward primer (same primers used for cloning PCR), Inqaba Biotec
- 100 µM reverse primer (same primers used for cloning PCR), Inqaba Biotec
- 2x Go Taq master mix, Thermo Scientific, USA

One colony of E. *coli* cells was inoculated in 20 µL water. The samples were boiled at 95 ºC for 10 min and then centrifuged at 13000 g for 1 min. After DNA extraction, the master mix for the colony PCR was prepared as shown in Table 2.7.

Table 2-7: Colony PCR master mix

Reagent	Final concentration in 25 µL
20 µM forward primer	0.4 µM
20 µM reverse primer	0.4 µM
2x Go Taq master mix	x1

The next step was to add 11.5 µL DNA to the master mix. The PCR was set up as follows: one cycle at 95 ºC for 2 min, 30 cycles at 95 ºC for 2 min, 55 ºC for 30 s, 72 ºC for 45 s, and a final cycle at 72 ºC for 5 min. After the PCR, a 2% agarose gel was set up for 1 h 45 min.

2.4.7 Plasmid extraction

Plasmids were selected for isolation using the Macherey-Nagel plasmid DNA purification kit based on the colony PCR results. Samples indicative of amplicons at the 650 bp region were used; this represents the length of the insert (target DNA). Before the purification of the plasmids, bacterial cells were cultivated and harvested. For cultivation of the bacterial cells, one colony of E. *coli* cells was inoculated in 1 mL LB broth and was then incubated overnight at 37 ºC on a heated shaker. After incubation, samples were centrifuged at 11000 g for 30 s. The supernatant was discarded and the pellets were lysed.

Thereafter, 250 µL buffer A1 was added to the pellet and it was resuspended by vortexing. After this, 250 µL buffer A2 was added to the resuspended cells and the mixture was gently mixed by inverting the tubes, between six and eight times. Samples were incubated at room temperature for up to 5 min or until the lysate appeared clear. Thereafter, 350 µL buffer A3 was added to the samples, which were then mixed by inverting the tubes between 6 and 8 times until the blue samples turned colourless. The samples were centrifuged at 11000 g for 5 min at room temperature.

After centrifugation, the supernatant was transferred into a NucleoSpin® Plasmid / Plasmid (NoLid) column, which was placed into a collection tube. The samples were centrifuged at 11000 g for 1 min. The flow-through was discarded, and the NucleoSpin® column was placed back into the collection tube. The silica membrane was washed with 600 µL buffer A4

(supplemented with ethanol) and centrifuged at 11 000g for 1 min. The flow-through was discarded and the NucleoSpin® column was placed back into the empty collection tube. Samples were centrifuged at 11 000g for 2 min, and the collection tubes were discarded. The NucleoSpin® column was placed into a 1.5 mL micro-centrifuge tube and 50 μL buffer AE was added. The samples were incubated for 1 min at room temperature and were then centrifuged at 11000 g for 1 min to elute the DNA. The plasmids that were isolated were sent to Inqaba Biotec for sequencing.

Chapter 3: Results

3.1 HPV DNA genotyping results

After genotyping analysis of 160 HPV mRNA positive samples, a total of 130 samples tested positive for HPV DNA. Six samples were invalid. Fifty-two samples were indicative of co-infections with other hrHPV sub-types. The percentage prevalence was calculated for each HPV sub-type using the total (N) of 154 samples, as summarised in Table 3.1.

Table 3-1: HPV DNA genotyping results

HPV sub-type	N positive (total N=154)	% positive (total N=154)
16	29	18.83
59	21	13.64
58	20	12.99
33	16	10.39
52	15	9.74
53	14	9.09
35	14	9.09
31	12	7.79
51	10	6.49
18	10	6.49
66	7	4.55
68	6	3.90
39	6	3.90
45	5	3.25
56	5	3.25
73	3	1.95
82	3	1.95
26	0	0
69	0	0

The HPV DNA genotyping results indicated that HPV type 16 was the most common HPV sub-type and was detected in 18.83% of the samples analysed. HPV type 59 was detected in 13.64%, making it the second-most common HPV type in this study. The hrHPV sub-types 58, 33, and 52 were detected in 12.99%, 10.39%, and 9.74% of the samples respectively. HPV 18 had a

relatively low prevalence at 6.49% and was ranked as the ninth-most common HPV sub-type in this study, along with HPV 51 (6.49%). The data are summarised in Figure 3.1.

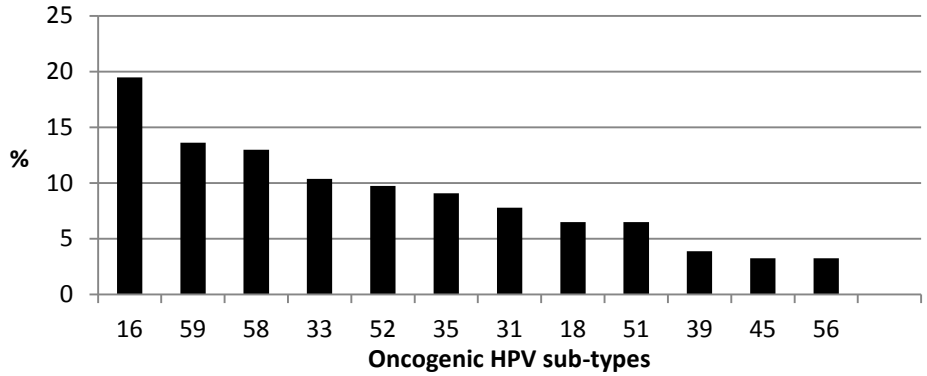


Figure 3-1: Prevalence of oncogenic hrHPV sub-types among the cohort. The prevalence of these 12 oncogenic HPV sub-types was analysed using Anyplex™ II HPV28 DNA genotyping assay.

In the following graphs, Figure 3.2 indicates the prevalence of other hrHPV genotypes, which was analysed with the HPV DNA genotyping kit from Seegene®. The prevalence of lrHPV types is summarised in Figure 3.3. Figure 3.4 is a representation of all the HPV genotypes detected by the HPV DNA genotyping kit from Seegene®.

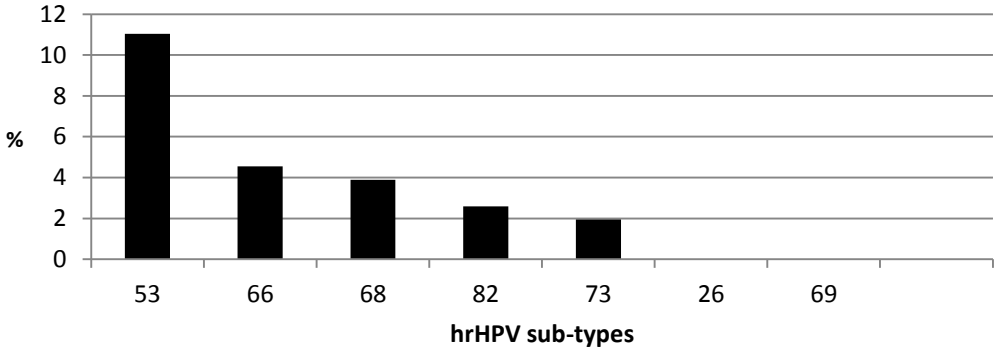


Figure 3-2: Prevalence of other hrHPV sub-types among the cohort. Prevalence was analysed using the Anyplex™ II HPV28 DNA genotyping assay.

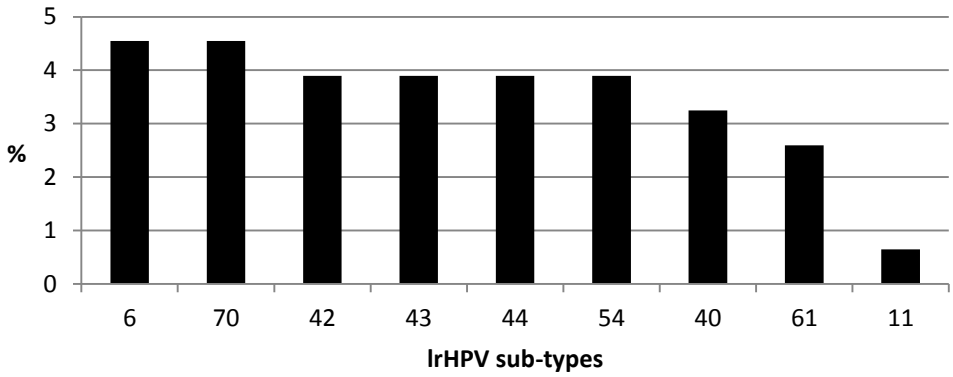


Figure 3-3: Prevalence of lrHPV sub-types among the cohort. Analysis was performed using the Anyplex™ II HPV28 DNA genotyping assay.

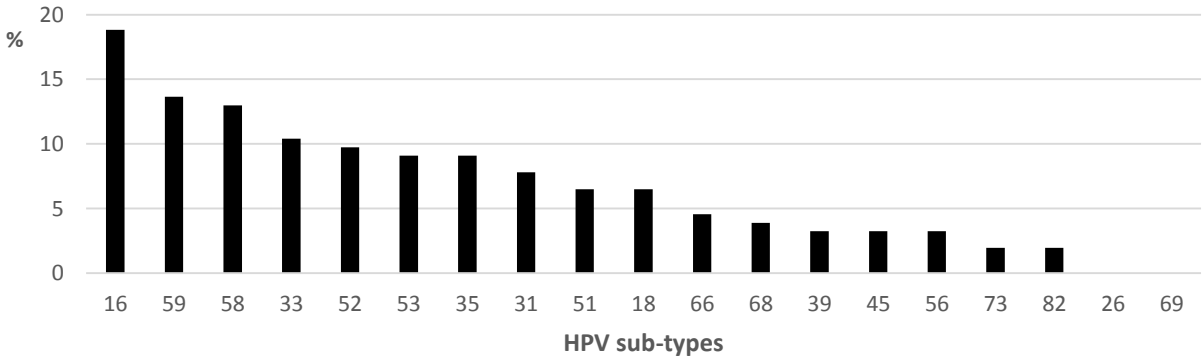


Figure 3-4: Overall HPV prevalence among the cohort. Prevalence of oncogenic, hrHPV, and lrHPV sub-types was analysed using the Anyplex™ II HPV28 DNA genotyping assay.

Figure 3.5 indicate cytology results that were available (N=112) for the samples from the cohort in this study.

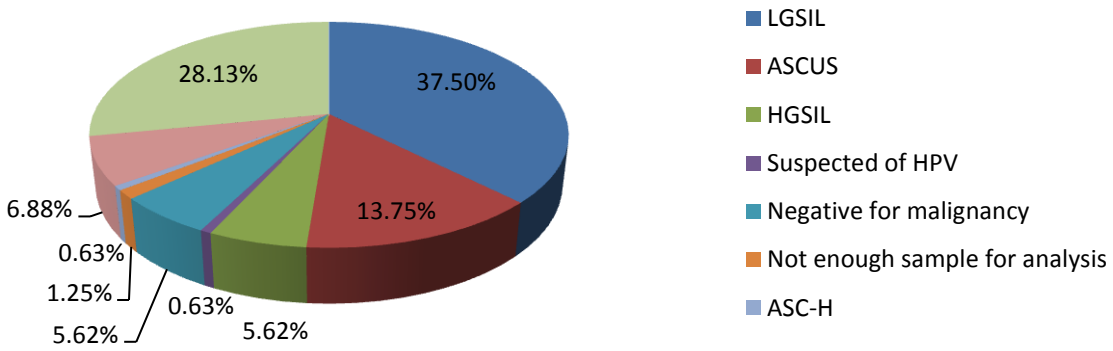


Figure 3-5: Cytology results for the cohort. Results for mRNA positive samples that had cytology results available (N=112). Cytology results included LGSIL, ASCUS, HGSIL, suspected of HPV, negative for malignancy, not enough sample for analysis, atypical squamous cells cannot exclude HGSIL, negative for endocervical transformation zone (EC/TZ) and cytology results not available (N/A).

There were 29 discordant results between the HPV mRNA and DNA genotyping assays. Among this group of samples, 24 were HPV DNA negative and five were negative for hrHPV DNA but positive for lrHPV sub-types (HPV sub-types 6, 44, 61, and 70). Cytology for 10 of the 24 samples that tested positive for HPV mRNA but negative for HPV DNA was indicative of ≤ LGSIL. Fourteen of the 24 samples had no cytology results available. Cytology results for the 5 HPV mRNA positive samples that were negative for hrHPV DNA but positive for lrHPV DNA were all indicative of ≤ LGSIL. Table 3-2 summarise the discordant results between the HPV mRNA and HPV DNA genotyping assays.

Table 3-2: Discordant results between the HPV mRNA and HPV DNA genotyping assays

Sample	hrHPV DNA Result	lrHPV DNA Result	Cytology
374259	Negative	Negative	ASCUS
377932	Negative	HPV 61	LGSIL
382621	Negative	Negative	LGSIL
464020	Negative	Negative	Not Available
459768	Negative	Negative	Not Available
387040	Negative	Negative	Not Available
387170	Negative	Negative	Not Available
387908	Negative	Negative	Not Available
388769	Negative	Negative	Not Available
389017	Negative	Negative	Not Available
389024	Negative	Negative	Not Available
389881	Negative	Negative	Not Available
395812	Negative	Negative	LGSIL
403185	Negative	HPV 6, HPV 70	LGSIL
405132	Negative	HPV 70	Not Available
301271321	Negative	Negative	Negative EC/TZ
518472	Negative	Negative	LGSIL
301224059	Negative	Negative	Not Available
301255641	Negative	Negative	Not Available
518338	Negative	Negative	Not Available
520120	Negative	HPV 70	ASCUS
301257963	Negative	Negative	Not Available
513835	Negative	Negative	Negative EC/TZ
518576	Negative	HPV 44	ASCUS
515457	Negative	Negative	Negative EC/TZ
513862	Negative	Negative	LGSIL
518471	Negative	Negative	ASCUS
515045	Negative	Negative	LGSIL
301209800	Negative	Negative	Not Available

After HPV DNA genotyping, twelve samples were sequenced. These samples represented the 12 known oncogenic (group 1) HPV sub-types, and they were sent for sequencing to investigate the variants of HPV sub-types that might be present in these samples. Sequencing results indicated that most samples had either mixed infections or no amplification although HPV DNA genotyping results were indicative of single infections. One of the samples used for sequencing

was positive for HPV sub-type 33 but sequencing results were indicative of HPV sub-type 67. Cloning was done to separate the mixed profiles and to sequence single infections.

3.2 Cloning results

All the samples initially sent for sequencing were used for the cloning PCR. Gel confirmation (Figure 3.6) of the PCR results showed that only HPV sub-types 33, 56, and 58 were amplified.

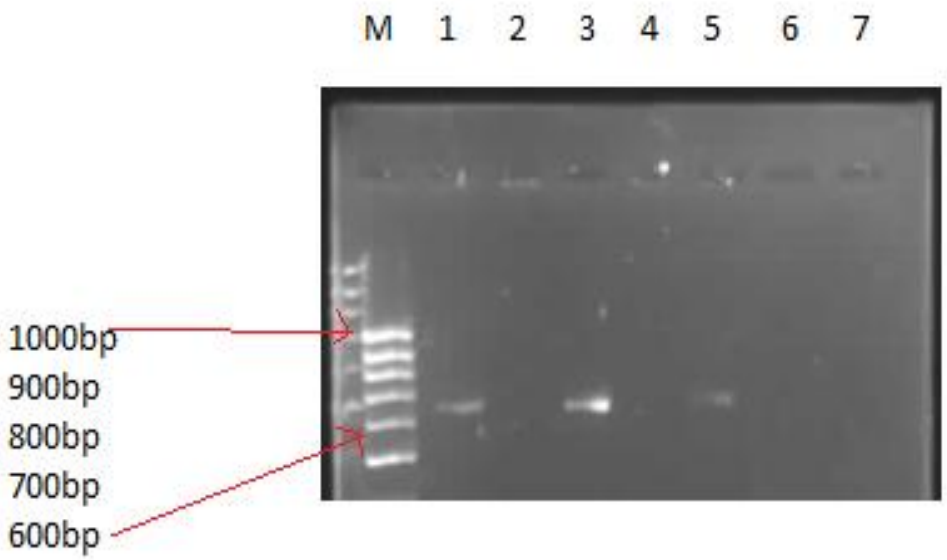


Figure 3-6: Cloning PCR results. Lane M was loaded with a 100 bp fragment length marker. This gel indicates fragment lengths of 650bp for HPV sub-types 33 (lane 1), 56 (lane 3), and 58 (lane 5), indicating that cloning was successful for these samples.

Only the 3 HPV sub-types that were amplified were used further for cloning. At the end of cloning, the plates containing HPV sub-types 33, 56, and 58 displayed blue and white colonies (Figure 3.7).

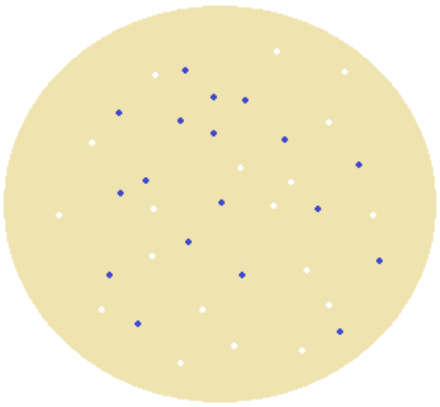


Figure 3-7: Schematic representation of the LB agar plates after cloning. The blue colonies represent E. *coli* cells with a plasmid that has no target DNA insert. The colourless colonies represent E. *coli* cells with a plasmid that has the target insert fragment. The colourless colonies were used for sequencing.

As previously explained, the white colonies are indicative of E. *coli* cells with a plasmid that has a target DNA insert, and these colonies were used for sequencing. The colony PCR gel showed amplification for HPV of both type 33 and type 58 (Figure 3.8).

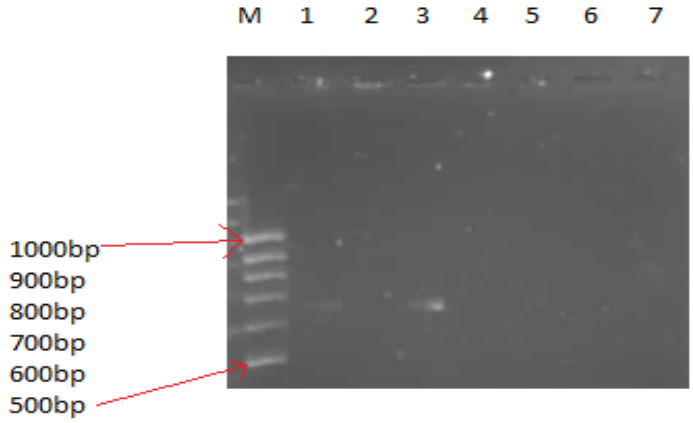


Figure 3-8: Colony PCR results. Lane M was loaded with a 100 bp fragment length marker. This gel indicates amplification fragment lengths of 650 bp for HPV 33 (lane 1) and 58 (lane 3), indicating that the colony PCR was successful.

A PCR digest reaction was performed to test whether the cloning was successful. The gel confirmation for the digest reaction was negative for both HPV sub-types, a result that indicated

cloning was not successful. It was decided, based on the colony PCR that HPV sub-types 33 and 58 would be sent for sequencing.

3.3 Sequencing results

Sequencing (shown in Appendix D) was successful for HPV 33, but HPV 58 had no amplification. A basic local alignment search tool (BLAST) on National Center for Biotechnology Information (NCBI) confirmed that the sequencing results for the sample that was positive for HPV 33 were again indicative of HPV sub-type 67.

Chapter 4: Discussion

McDonald *et al.* (42) stated that HPV 16 was less prevalent in Africa than the rest of the world, although a study conducted by McDonald *et al.* (42) among HIV negative women with or without CIN from the Western Cape, Cape Town, indicated that HPV 16 was the most prevalent HPV type detected in their study. Results for this study on HIV unknown women with or without CIN results from Gauteng and Limpopo provinces, indicated that HPV type 16 was the most common HPV sub-type and was detected in 18.83% of the samples analysed. A study conducted by van Aardt *et al.* (40) among peri-urban women without cytological abnormalities in the Tshwane district of Gauteng, South Africa, also indicated that HPV type 16 was the most prevalent HPV type detected in their study. HPV type 18 had a low prevalence of 6.49% and was ranked as ninth commonest HPV sub-type in this study, along with HPV 51. HPV sub-types (in descending order) 59, 58, 33, 52, 35 and 31 were all more prevalent than HPV 18. Even though HPV 16 and 18 are the most prevalent HPV sub-types in the world (35,43-46), type-specific prevalence differences within each disease grade have also been observed by Bzhalava 2013 *et al.* (32) and Smith *et al.* (59).

There were discordant results between the Aptima HPV E6/E7 mRNA and Anyplex™ II HPV 28 DNA genotyping assays. Possible reasons for the discordant results include: different targets of amplification, possible mRNA contamination, cross-reactivity of the HPV E6/E7 mRNA assay with lrHPV sub-types and the quantity of DNA extracted.

As mentioned already HPV genotyping with the Anyplex™ II HPV 28 DNA genotyping kit relies on detection of the L1 gene (56). The L1 gene becomes disrupted and downregulated if viral integration occurs (60-61). This fact renders detection of HPV DNA difficult in assays that only detect the L1 region. However, for this specific study it cannot be assumed that the

disruption or downregulation of the L1 gene is responsible for the discordant result because there is not enough evidence. There is no histological evidence to confirm the severity of cervical dysplasia and there were no follow-up tests done on the patients samples.

Another explanation for the discordant results could be mRNA carry over contamination during processing of the samples. Unfortunately, the samples send for routine HPV E6/E7 mRNA screening were not retested because contamination was not expected at the time of testing. It is standard operating procedure for the laboratory staff at the molecular laboratory of Vermaak and Pathcare to include negative calibrators on every run. Since the calibrators were negative no contamination was suspected.

Good quality DNA extraction is important for the sensitivity of HPV DNA detection. The LBC sample volume used for extraction of HPV DNA was 200 µL. The LBC sample volume used for the Aptima HPV E6/E7 mRNA assay was 1000 µL. The difference in sample volume used could influence the sensitivity of the DNA test and could therefore explain the HPV mRNA positive but HPV DNA negative samples. As mentioned already, the Anyplex™ II HPV28 detection kit amplifies beta globin as an internal control. Even though the internal control was detected in all the samples that tested positive for HPV mRNA but negative for HPV DNA, the volume used for extraction could still influence the sensitivity of the detection kit especially for samples that had a low HPV viral load. This was also observed by Lillsunde *et al.* (62).

The discordant samples that were HPV mRNA positive, hrHPV DNA negative but lrHPV DNA positive could be false positivity due to cross-reactivity to untargeted, low risk genotypes (63). Cross-reactivity has been described in literature for the Aptima HPV E6/E7 mRNA assay (63-64). Gibson *et al.* (64) described untargeted amplification and detection of HPV 26, HPV 67,

HPV 70 and HPV 82 leading to false positive results. Preisler *et al.* (63) described untargeted amplification and detection of HPV 61, 62, 70, 82 and 83. The Aptima HPV E6/E7 mRNA assay package insert indicate untargeted amplification of lrHPV types 26, 67, 70, and 82 leading to false positive results (63). Four of the 5 HPV mRNA positive, hrHPV DNA negative but lrHPV DNA positive results were indicative of HPV 61 or HPV 70.

To investigate the HPV variants present in the samples used for this study, 12 samples indicative of single HPV infections were sent for sequencing. Only two samples were sequenced successfully; the other samples had either mixed profiles or no amplification. The mixed profiles observed during sequencing could be caused by non-specific primer binding by the degenerate primers used for sequencing. Another explanation is that the threshold set for the HPV DNA genotyping assay on the CFX analyser could have been set too high to detect other types, which may have had low amplification and were considered as background information by the instrument. No amplification could be the cause of DNA degradation. One sample tested positive for both HPV mRNA and HPV type 33 DNA, but sequencing results for this sample were indicative of HPV type 67. This result could be explained by non-specific primer binding, higher sensitivity of sequencing, or false mRNA positivity due to cross-reactivity as described by Gibson *et al* (64).

The high sensitivity and high throughput of HPV molecular screening are reasons for the choice of this screening strategy for cervical cancer caused by HPV. According to Chan *et al.* (61) the choice of the HPV test depends on the application. Molecular epidemiological studies and evaluating vaccine efficacy studies require assays with high analytical sensitivity (61). HPV typing assays with high analytical sensitivity and specificity can be used for virological surveillance, including the evaluation of vaccination impact on the prevalence of vaccine-

covered types, identification of new types, discrimination of types in multiple infection, and monitoring of potential type replacement in the post-vaccine era (61). Since most HPV infections clear without inducing cell dysplasia, HPV-testing struggles with clinical specificity (62). Therefore assays with lower analytical sensitivity may produce a better positive predictive value (PPV) for cervical neoplasia and will be more effective in cervical cancer screening and post-treatment follow-up (63).

HPV DNA testing is very sensitive for the detection of HPV, but this high sensitivity leads to a low PPV of 15-25% for high-grade lesions by biopsy (33). With regard to HPV DNA genotyping additional genotype information is valuable (3) as the risk of developing neoplasia may vary with different HPV sub-types (55). Smelovet *et al.* (65) conducted a long-term study over 14 years; the results showed that the cumulative incidence for CIN3 in women infected with the hrHPV types 16, 18, 31, and 33 was above 28% (55). Therefore, information on specific genotypes could be important for patient treatment and follow-up (66-68).

In persistent infections there is overexpression of E6/E7 mRNA, and therefore the infection is less likely to regress (15). Detection of overexpression of E6/E7 mRNA may be more directly associated with disease progression (15). Although HPV mRNA testing is more specific in predicting the presence of dysplasia when compared to HPV DNA testing, a weakness of HPV mRNA testing is that it is less sensitive in detecting the presence of HPV latent infections (69).

Certain cofactors are associated with increased risk for persistence and integration by HPV (33). These cofactors include HIV co-infection, tobacco smoking, high parity, and long-term hormonal contraceptive use (33). Co-infection with *Chlamydia trachomatis* and *Herpes simplex* virus type 2, immunosuppression, and certain dietary deficiencies have been identified as

possible cofactors (33). Patients' age and sexual activity influence screening intervals and treatment strategies too (48).

Chapter 5: Conclusion

HPV DNA genotyping results indicated that HPV 16 with a result of 18.83% was the most common HPV sub-type detected and this prevalence of HPV 16 was consistent with findings reported in the literature. The 10 most prevelant HPV sub-types detected in this study were all from group 1A carcinogens, except for one, HPV 35, that was detected as 6 commonest HPV sub-type with a percentage of 9.09 %.

Considering the advantages and disadvantages of HPV DNA and HPV mRNA assays, the cofactors that influence screening strategies for cervical cancer, the fact that the prevalence of different HPV sub-types varies among diverse populations and type-specific prevalence differences within each disease grade, triage testing as recommended by the HPV advisory board is probably the most important step in cervical cancer screening.

Considering the number of discordant results in this study it is imperative that HPV prevalence studies must be standardized before actual testing. Factors that can influence the sensitivity of HPV testing must first be optimized. An example is the extraction method used. As observed in this study the sample volume used could possibly influence the sensitivity of the assay.

It is important to include as much information as possible for the cohort used in a study because it can be used to explain discordant results. For instance, histological information could have explained mRNA positive but DNA negative results as seen in this study.

Even though the aim of this study did not focus on the effectiveness of vaccines in South Africa and only 160 samples were used for this study, results indicate that the new 9vHPV vaccine

would be beneficial for South Africa because it vaccinate against 4 of the 5 most prevalent HPV sub-types in this study.

Appendix A

Table A: HPV DNA epidemiology of 160 HPV mRNA positive samples

Sample Number	**Oncogenic HPV Sub-types**	**hrHPV Sub-types**	**Low-risk HPV Sub-types**	**Cytology**
364122	16, 59		6	LGSIL
365399	39	66	40, 42	N/A
365668	33			LGSIL
366151	16		6	Negative
366201	31, 51, 58		42,54	LGSIL
366202	16			N/A
367341	56		43	LGSIL
367393	18, 52			ASCUS
367983	33	66		ASCUS
368915	45, 16			LGSIL
368936	16			HGSIL
369112	18, 31, 35, 58	53		N/A
369943	16			Negative
370179	33		54, 70	LGSIL
370180	16			ASCUS
370347	58			LGSIL
370348	16			LGSIL
370373	39			LGSIL
370823	16			LGSIL
371757	58		44	ASCUS
372873	16, 59	73	61	ASCUS
373042	31, 39			LGSIL
373990	16, 18, 31			LGSIL
374259	Negative	Negative	Negative	ASCUS
374260	18, 35, 51			LGSIL
374647	16			HGSIL
375540	58			LGSIL
375916	58			Negative
376559	16			Negative
377279	31, 52			LGSIL
377932			61	LGSIL
377933	33, 51	66		LGSIL
378114	58			ASCUS
378900	58			CIN1
378916	16, 35, 45		6, 54	LGSIL
380356	51	66		LGSIL

382621	Negative	Negative	Negative	LGSIL
382623	52	82		LGSIL
382746	33			LGSIL
384004		53	70	ASCUS
384639	45			LGSIL
384849	16, 35	68	6	LGSIL
384850	33, 51, 52		6, 40, 42, 43, 44, 61	LGSIL
385157	35			HGSIL
464016	58			N/A
464020	Negative	Negative	Negative	N/A
459768	Negative	Negative	Negative	N/A
460046	16			N/A
458558	52			N/A
456170	59		54	N/A
385159	52	73		LGSIL
385160	56			LGSIL
385563	16			ASCUS
386211		53, 68		LGSIL
386212	16			HGSIL
386427	51, 52	53		Negative EC/TZ
386428	16			LGSIL
386869		68	70	LGSIL
387040	Negative	Negative	Negative	N/A
387170	Negative	Negative	Negative	N/A
387290	52	68		LGSIL
387391	39			ASC-H
387756	33			N/A
387903	18		6	N/A
387908	Negative	Negative	Negative	N/A
388768	51			N/A
388769	Negative	Negative	Negative	N/A
388824	18			Negative
388967	33			N/A
389013	58			N/A
389017	Negative	Negative	Negative	N/A
389024	Negative	Negative	Negative	N/A
389331	56, 59		43	LGSIL
389332	59			LGSIL
389334	59			LGSIL
389335	16,51	53		LGSIL
389812	35, 39			ASCUS
389813	59			ASCUS
389881	Negative	Negative	Negative	N/A

390671	45, 58	53	44	Negative EC/TZ
391068	31			N/A
391412	16			N/A
391891	39, 58			LGSIL
392135	33, 35		40	HGSIL
392359	35, 45, 56, 59	73	40, 42, 43	LGSIL
392402	31, 35	53		LGSIL
392403	16, 33		11, 40	HGSIL
392466	16, 59			N/A
393216	16			LGSIL
393505	33, 59			LGSIL
393933	31, 35, 59			N/A
394277	35			N/A
394755	31			Negative
394756	35, 52, 59			LGSIL
394892	51	68		ASCUS
394893	52			HGSIL
395812	Negative	Negative	Negative	LGSIL
396172	58	53		LGSIL
397375	51	82	42, 44	LGSIL
400494	59			Negative
400600	16		43, 44	LGSIL
400801	33			Negative
401256	58			Negative EC/TZ
401257	31			N/A
401718	31			Suspected HPV
402717	59	66		Invalid
403185			6, 70	LGSIL
403299	59			N/A
403742	52			N/A
404551	Invalid	Invalid	Invalid	ASCUS
404832	33		42	Negative EC/TZ
405132			70	Invalid
405684	52, 58			ASCUS
406724	16	53,68, 82	54	LGSIL
406740		66		N/A
407168	59	53, 66	61	LGSIL
407475	58	53		LGSIL
407477	56	53	43	LGSIL
407478		53, 82		LGSIL
407681	16			Negative EC/TZ
408977	59		54	Negative EC/TZ
404370	59	53		LGSIL

467960	52			N/A
468673	52, 59	53		N/A
470547	18, 33			N/A
472907	35, 59			N/A
301271321	Negative	Negative	Negative	Negative EC/TZ
514832	33	53		LGSIL
518472	Negative	Negative	Negative	LGSIL
301224059	Negative	Negative	Negative	N/A
515181	58			Negative EC/TZ
301255641	Negative	Negative	Negative	N/A
518161	Invalid	Invalid	Invalid	LGSIL
518338	Negative	Negative	Negative	N/A
520120			70	ASCUS
516463	Invalid	Invalid	Invalid	ASCUS
515703	58			LGSIL
512878	18			HGSIL
518339	16			N/A
515265	Invalid	Invalid	Invalid	ASCUS
519527	Invalid	Invalid	Invalid	ASCUS
301257963	Negative	Negative	Negative	N/A
513610	58		70	Negative EC/TZ
301217273	59			N/A
513835	Negative	Negative	Negative	Negative EC/TZ
518576			44	ASCUS
515457	Negative	Negative	Negative	Negative EC/TZ
430008798	58			N/A
518786	33			ASCUS
514188		53		ASCUS
513862	Negative	Negative	Negative	LGSIL
518162	16			HGSIL
518471	Negative	Negative	Negative	ASCUS
301254258	Invalid	Invalid	Invalid	N/A
515045	Negative	Negative	Negative	LGSIL
301209800	Negative	Negative	Negative	N/A
518208	18			LGSIL
102109836	18, 35			N/A
102111853	16			N/A
518151	31, 52			Negative

Appendix B

The concentration of DNA in the samples was measured because some of the samples did not amplify during sequencing. The results obtained from the Nano drop, summarised in table B, showed DNA concentrations of between 222.02 ng/µl and 713.82 ng/µl at 260 nm in the samples.

Table B: Nano drop results

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280		260/230		Constant		Cursor
hpv16	Default	2016/07/18	09:43 AM		266.46	5.329	1.619	3.29	3.57	50.00	230	1.494	0.024
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hpv51	Default	2016/07/18	09:52 AM		336.01	6.720	2.105	3.19	3.43	50.00	230	1.957	0.392
hpv52	Default	2016/07/18	09:53 AM		246.08	4.922	1.573	3.13	3.44	50.00	230	1.430	0.092
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hpv58	Default	2016/07/18	09:55 AM		239.86	4.797	1.506	3.19	3.33	50.00	230	1.439	0.085
hpv59	Default	2016/07/18	09:56 AM		306.39	6.128	1.865	3.29	3.65	50.00	230	1.677	0.098

Appendix C

After the inoculation of the transformed *E. coli* cells, blue and white colonies were observed on the plates. The white colonies were picked off and inoculated onto new plates. The growth of theses *E. coli* cells are shown in figure C.1-C.3.

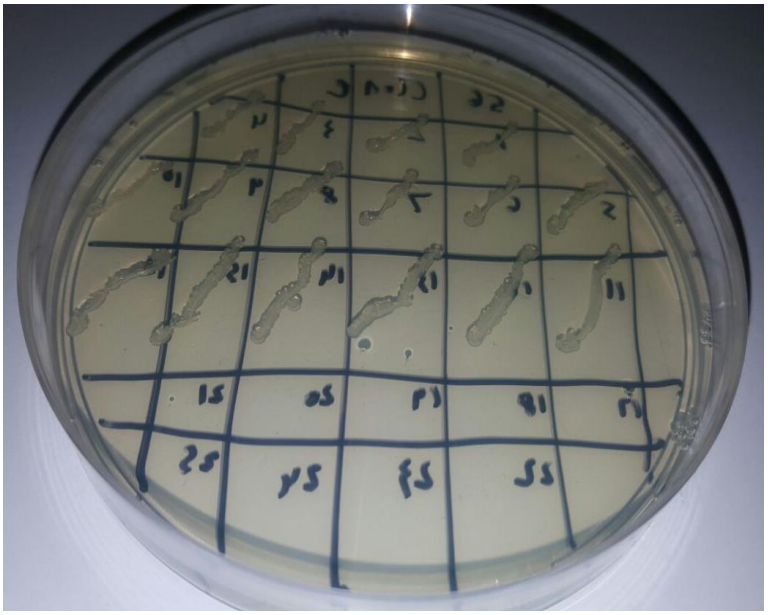


Figure C.1: HPV 56 agar plate. Figure C.1 indicate the growth of the white colonies that were picked of the inoculated transformed *E. coli* cells for HPV 56.

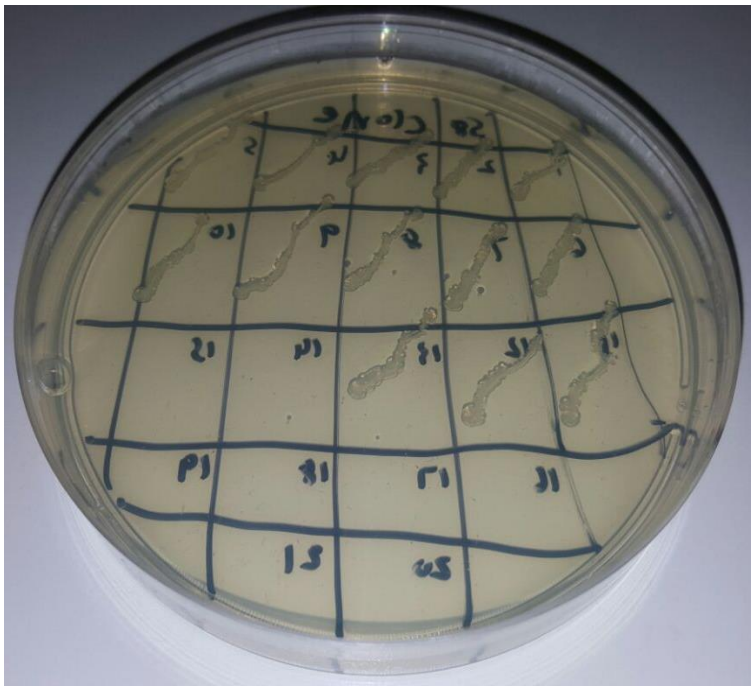


Figure C.2: HPV 58 agar plate. Figure C.2 indicate the growth of the white colonies that were picked of the inoculated transformed *E. coli* cells for HPV 58.

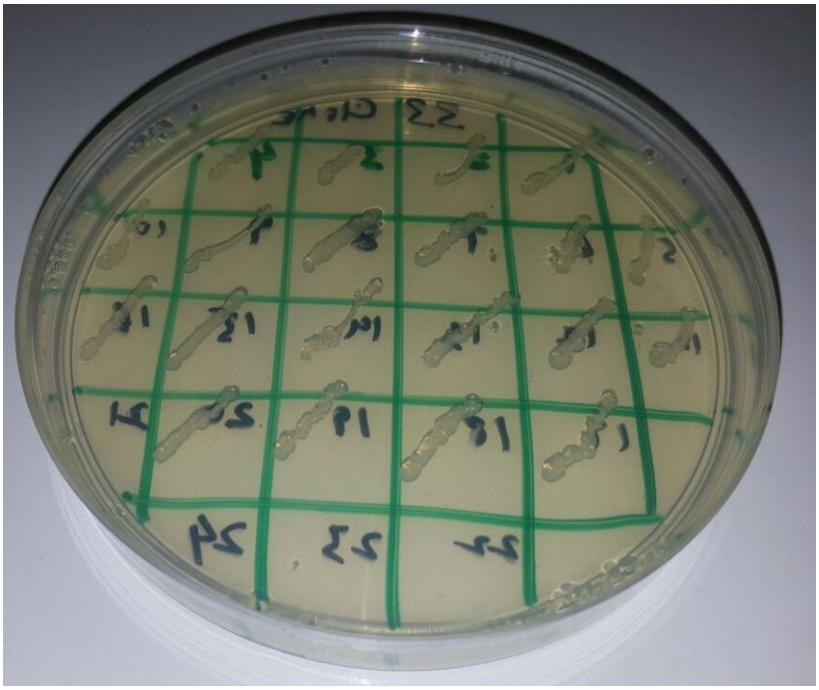


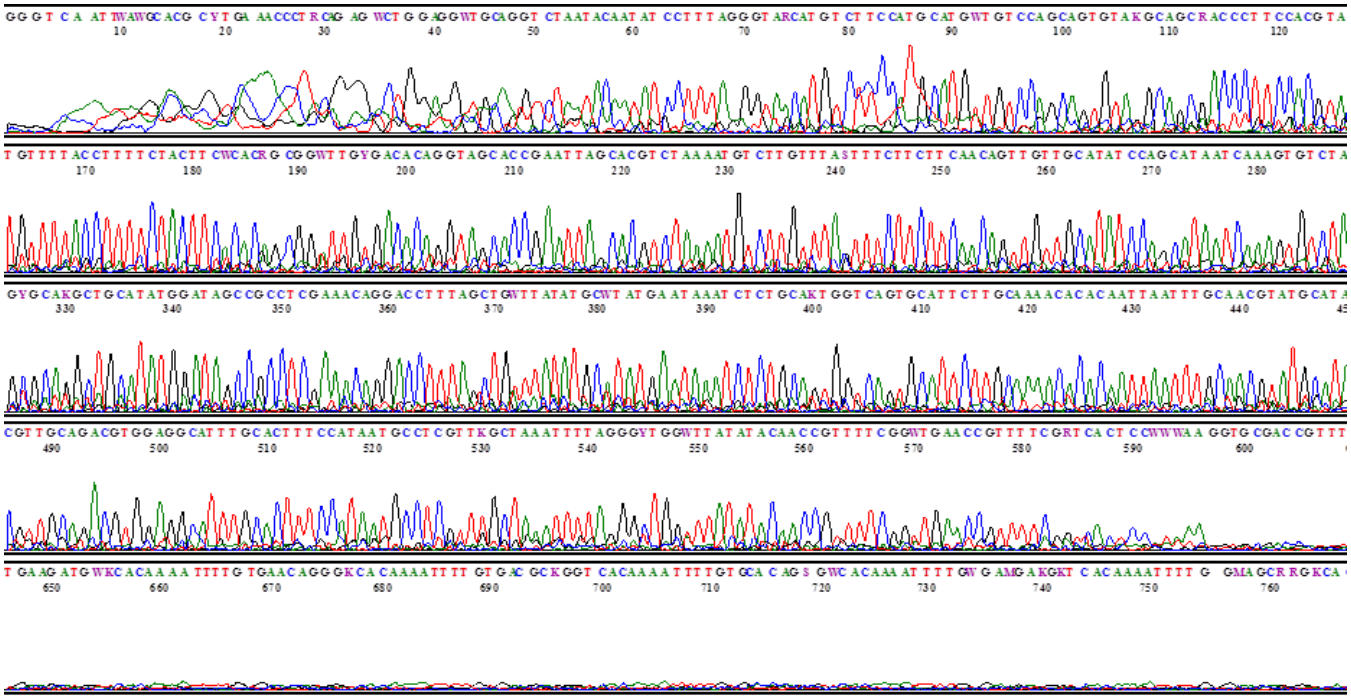
Figure C.3: HPV 33 agar plate. Figure C.3 indicate the growth of the white colonies that were picked of the inoculated transformed *E. coli* cells for HPV 33.

Appendix D

Sequencing results of the samples send to Inqaba for sequencing.

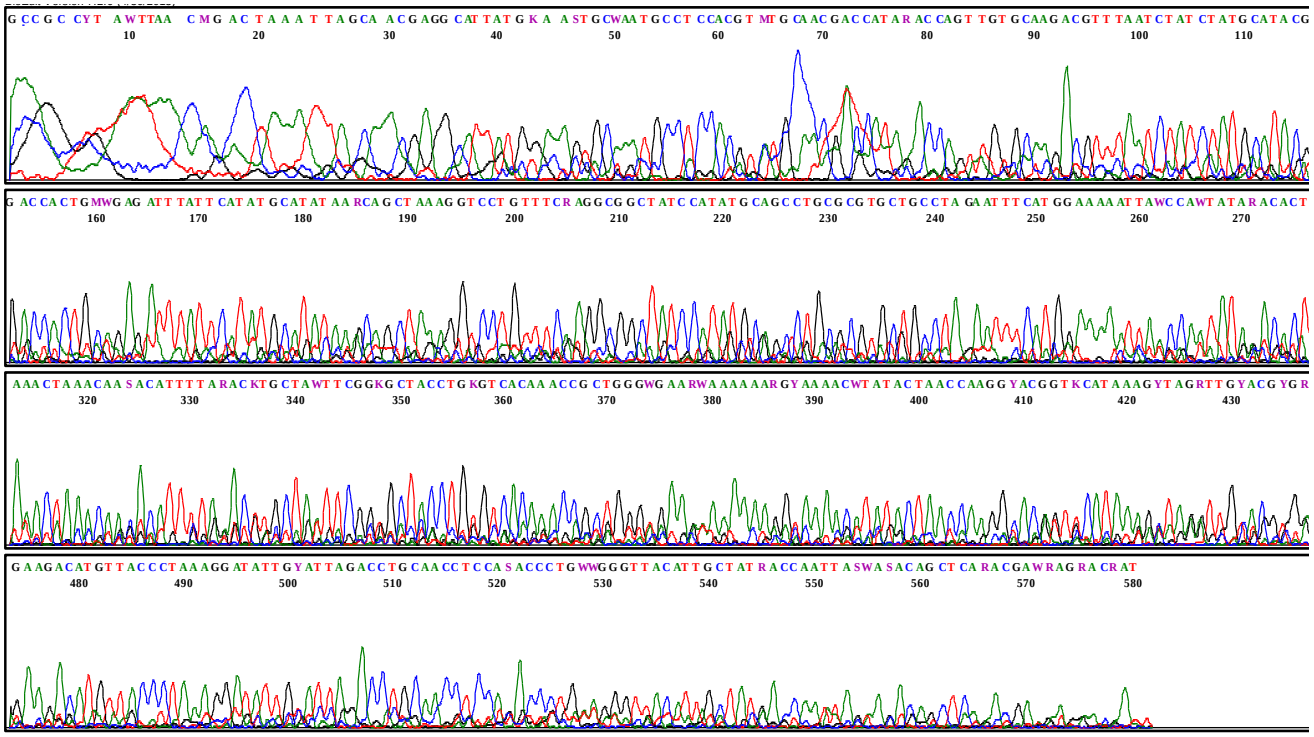
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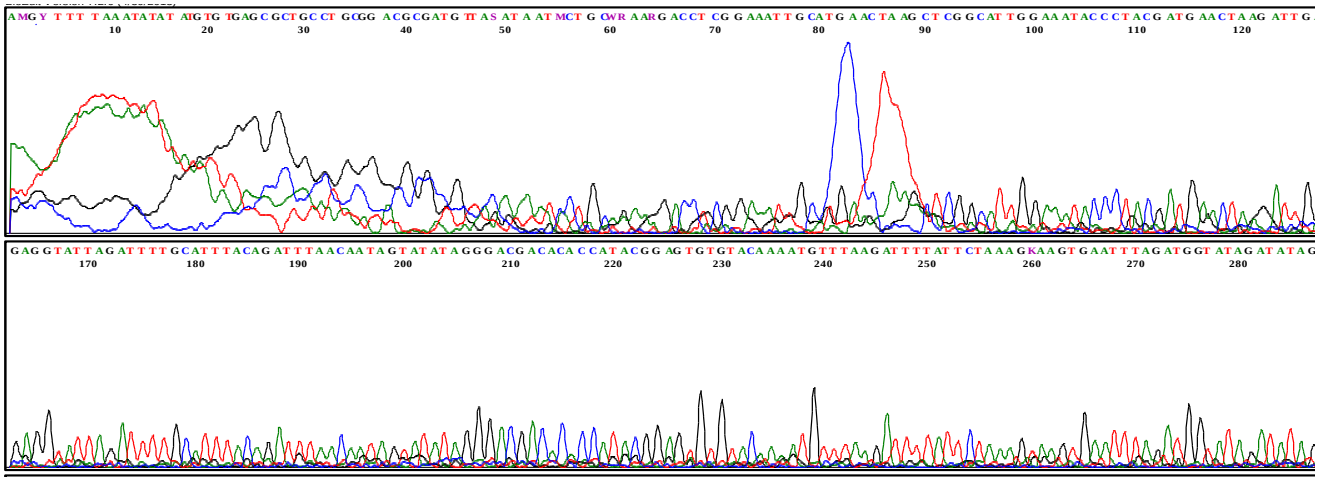
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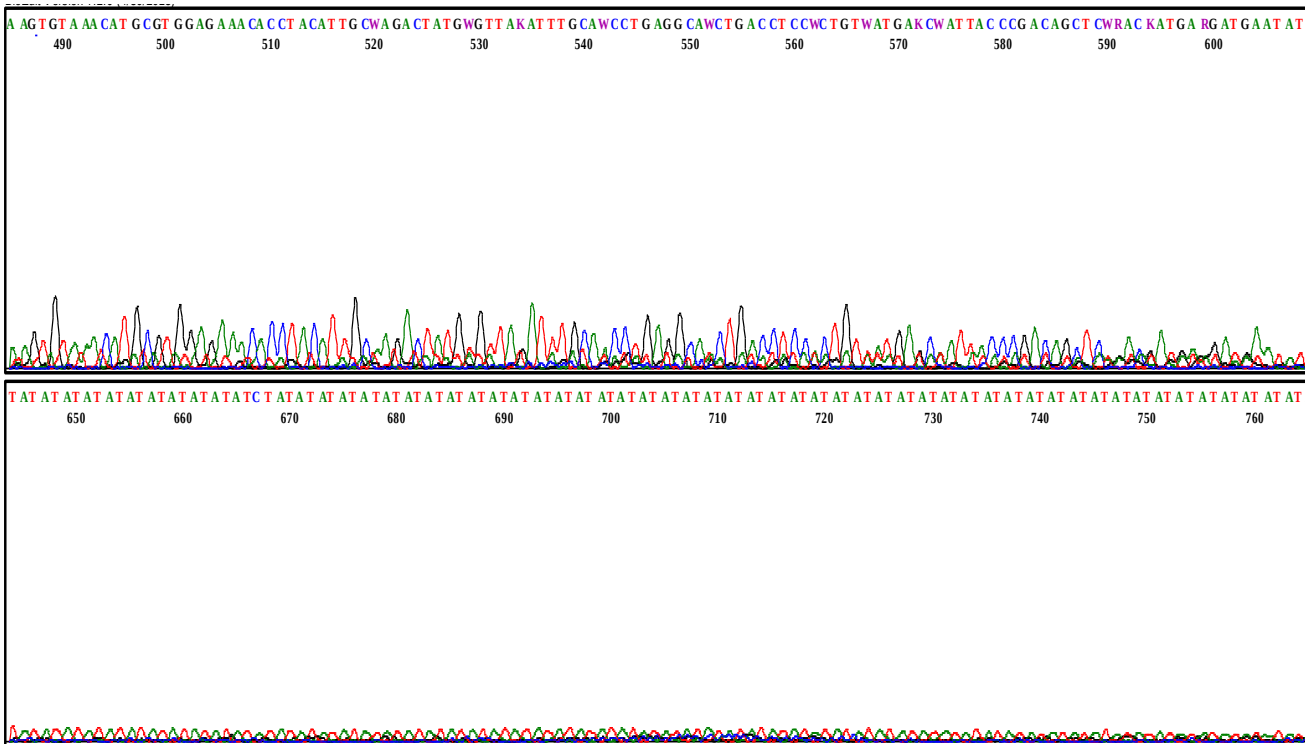


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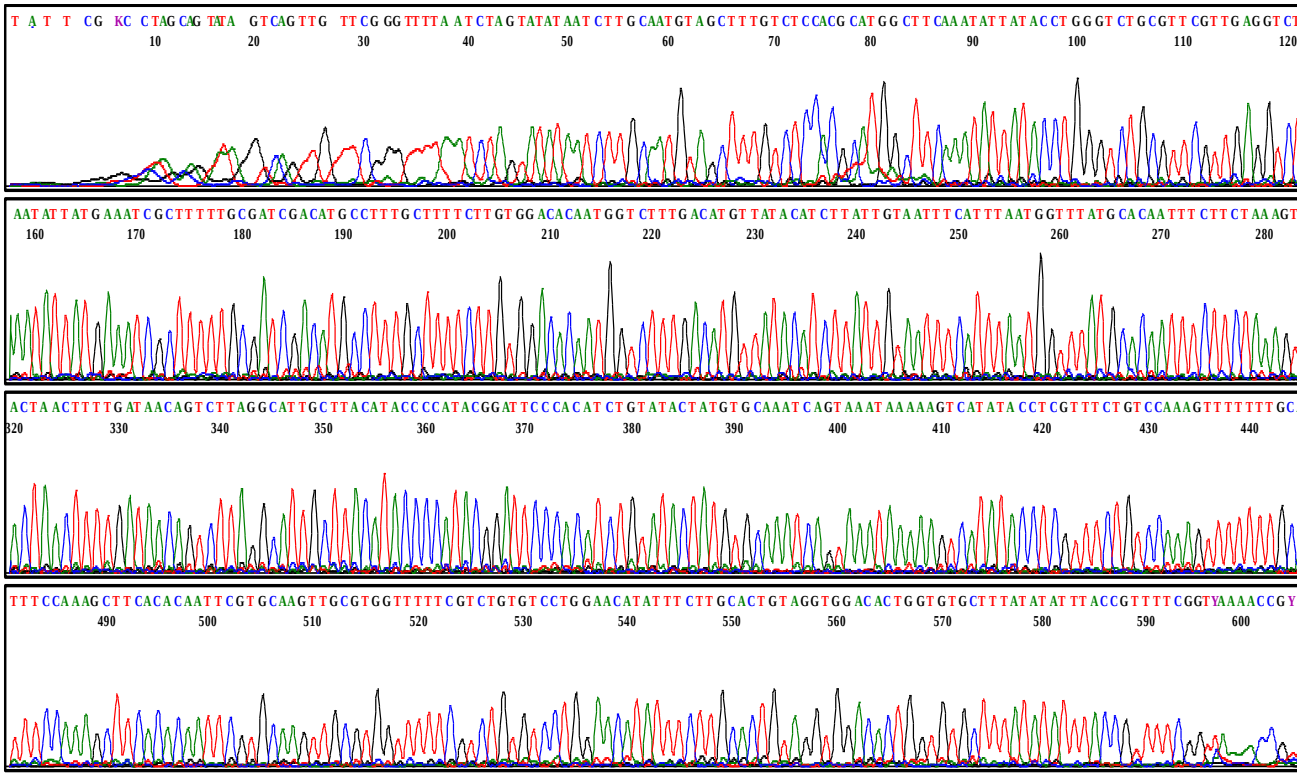
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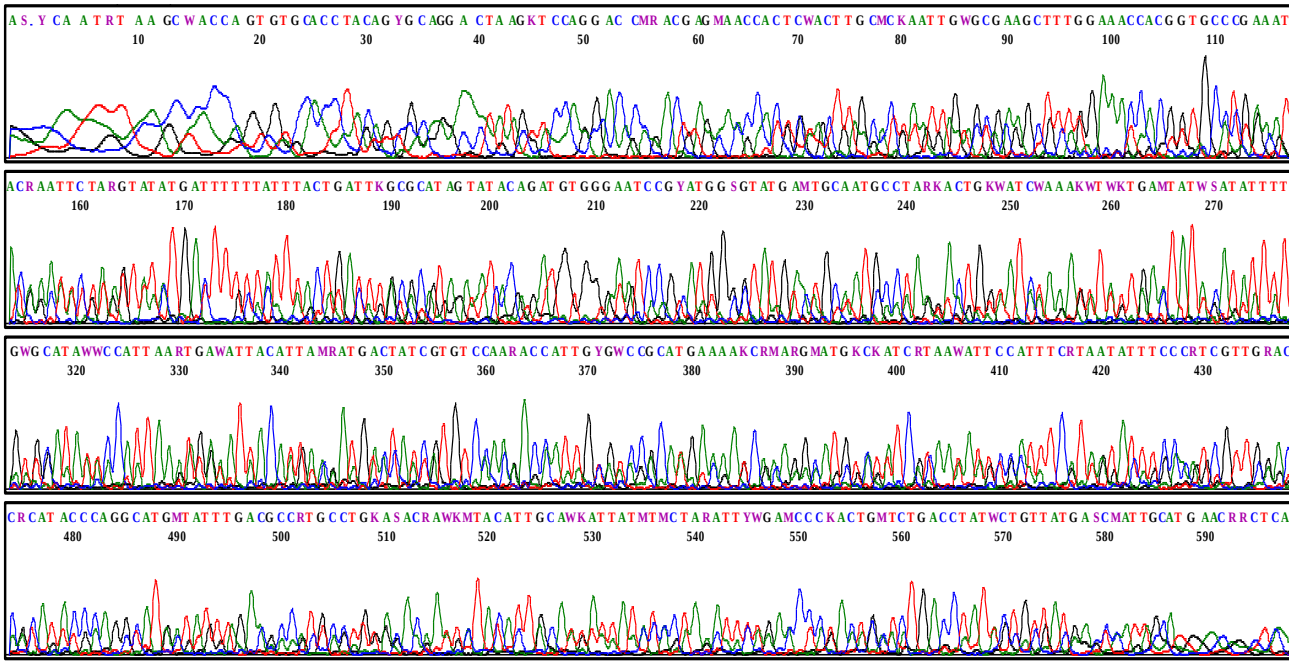
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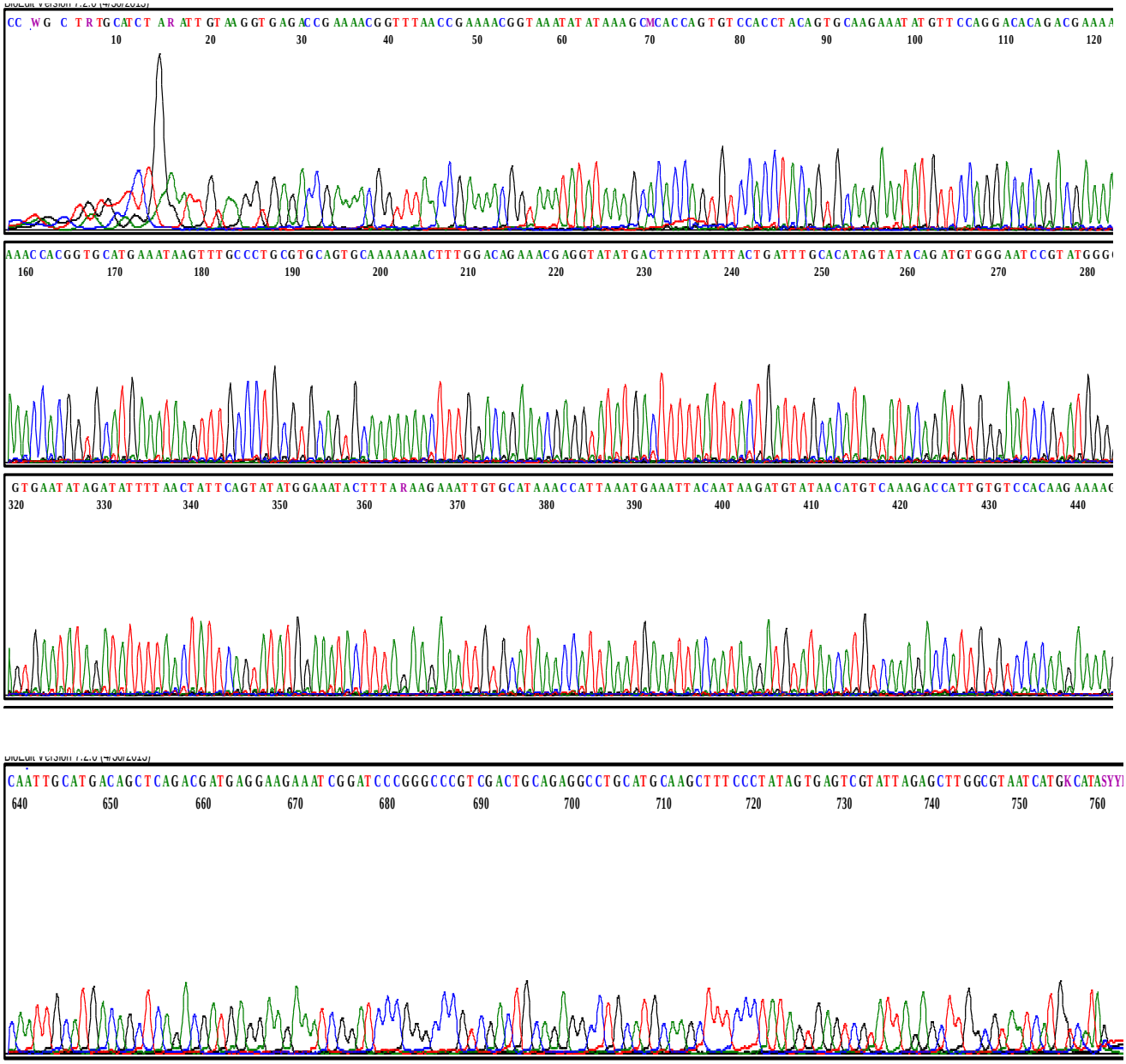
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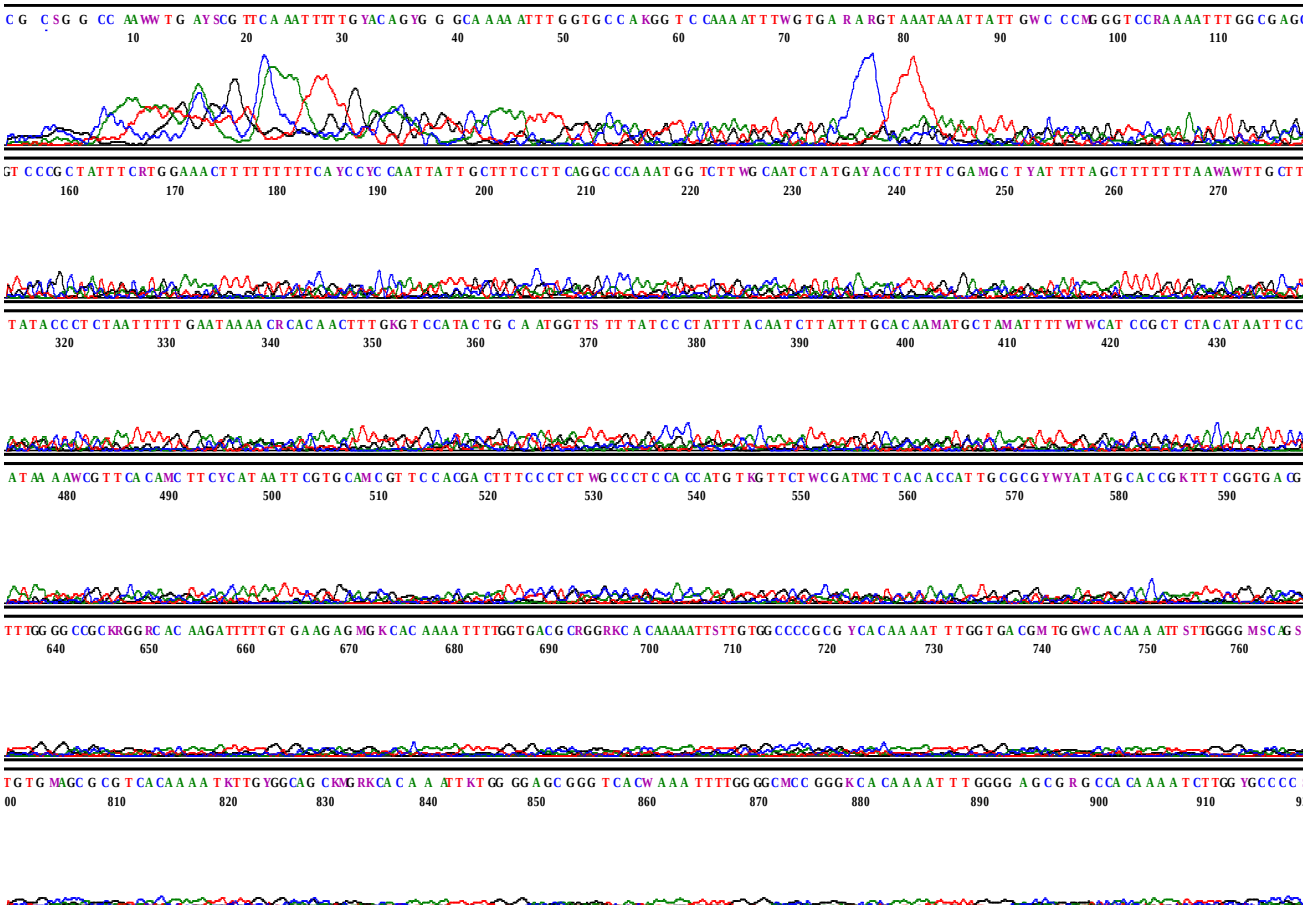
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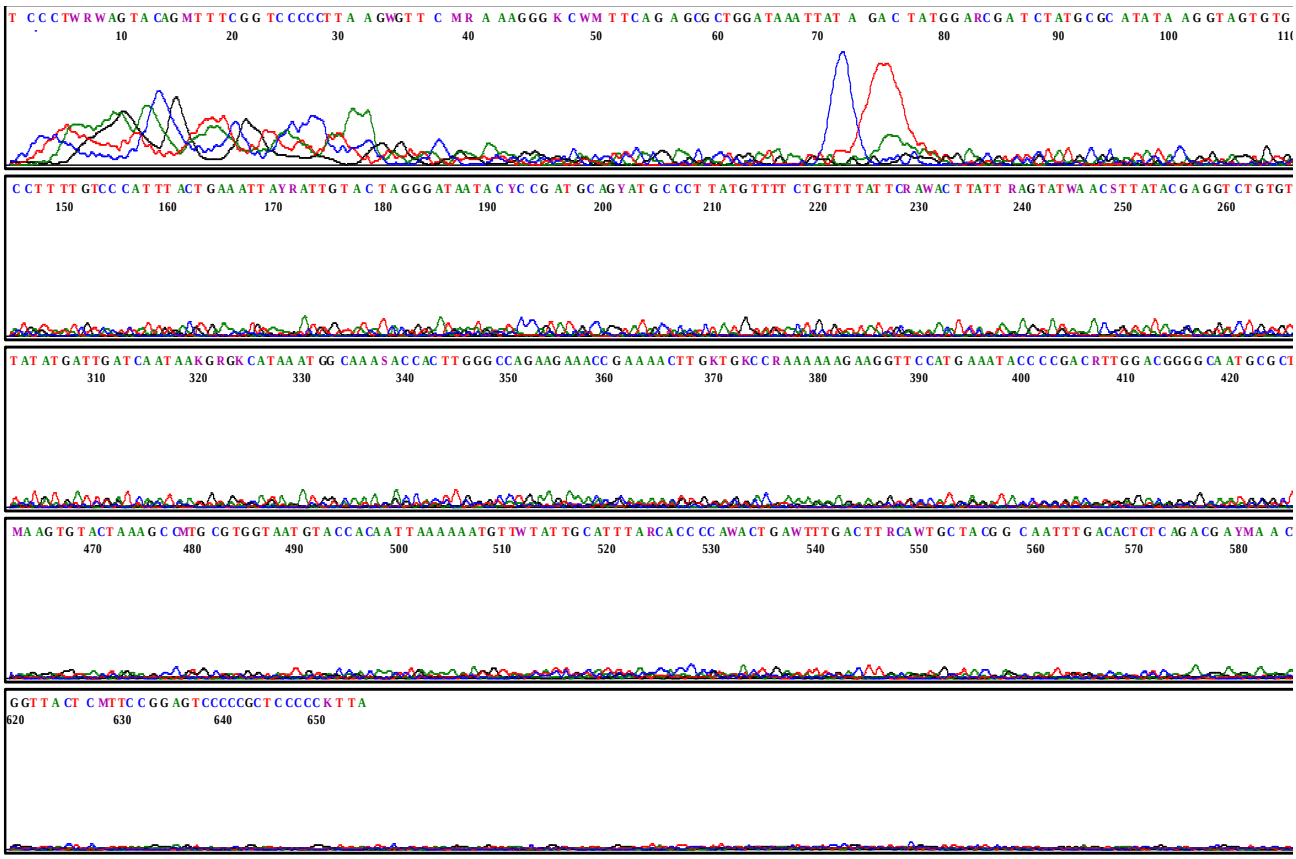
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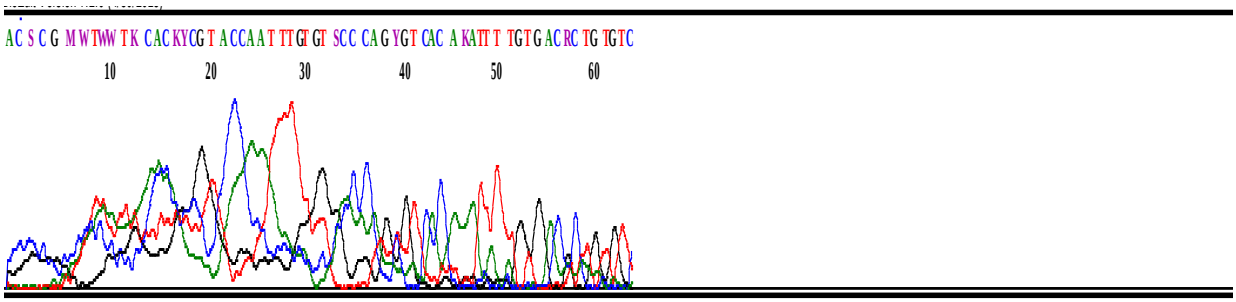
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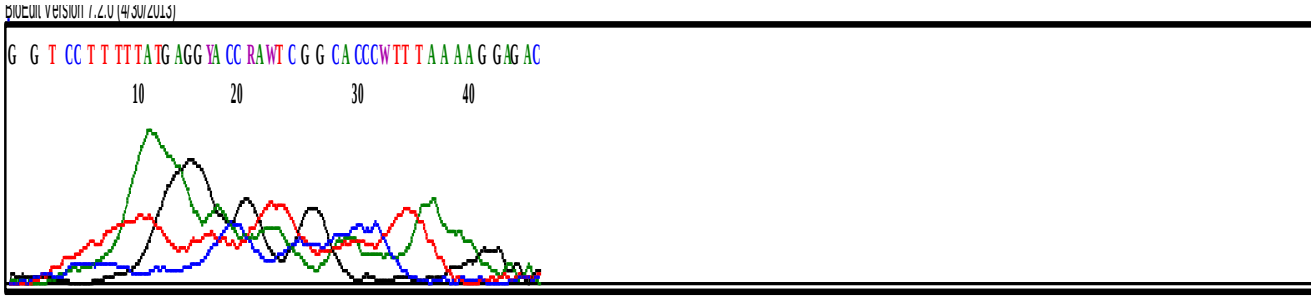
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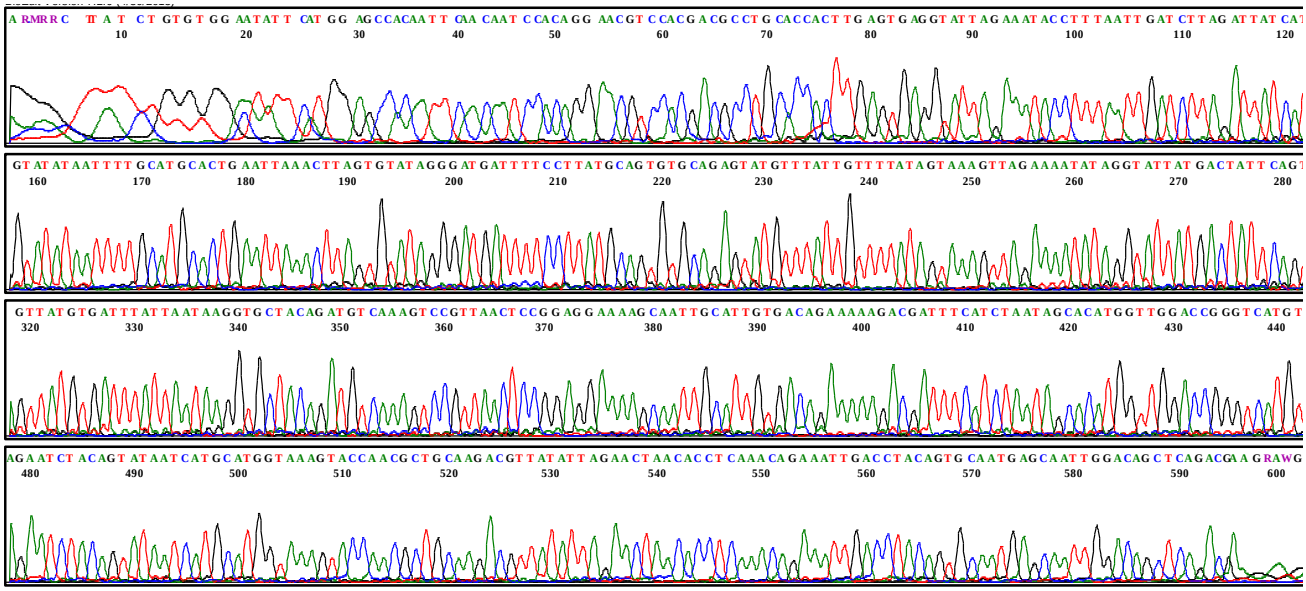
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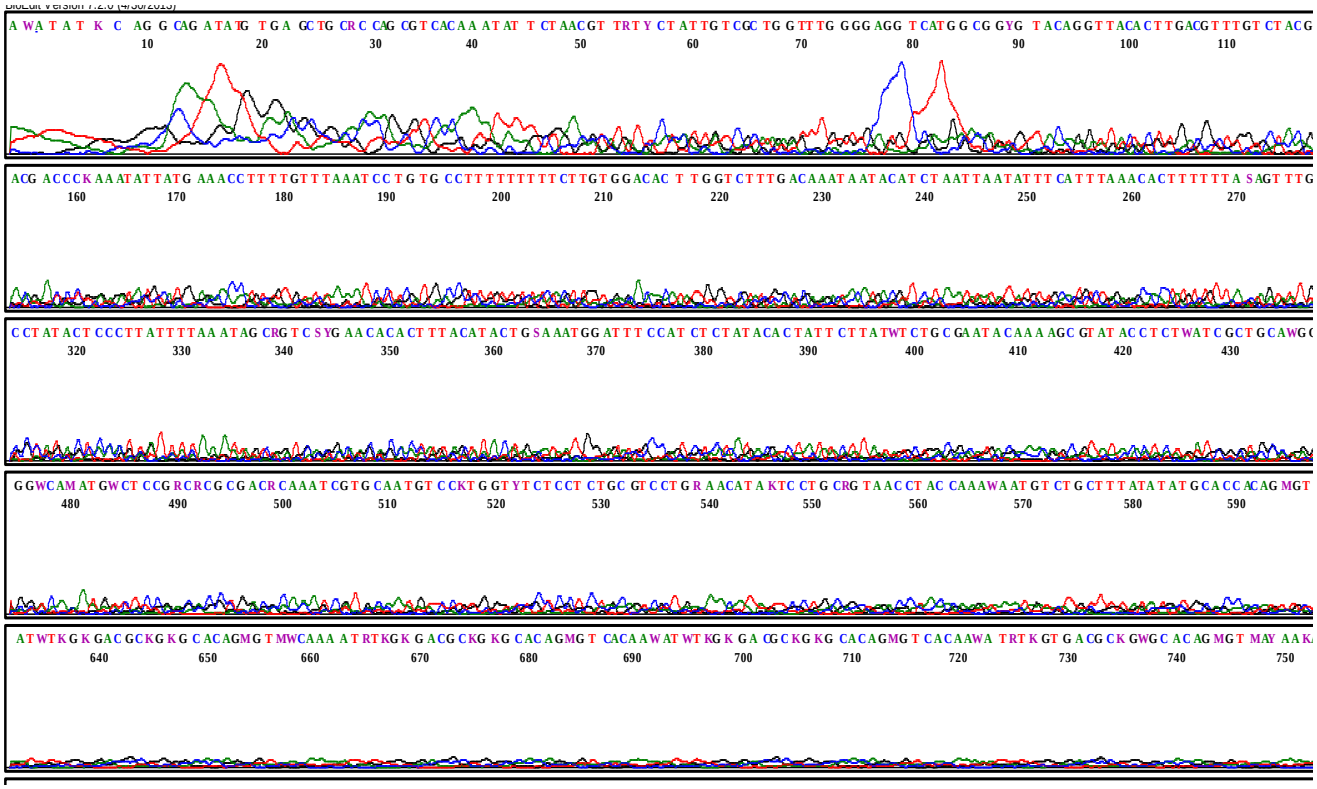
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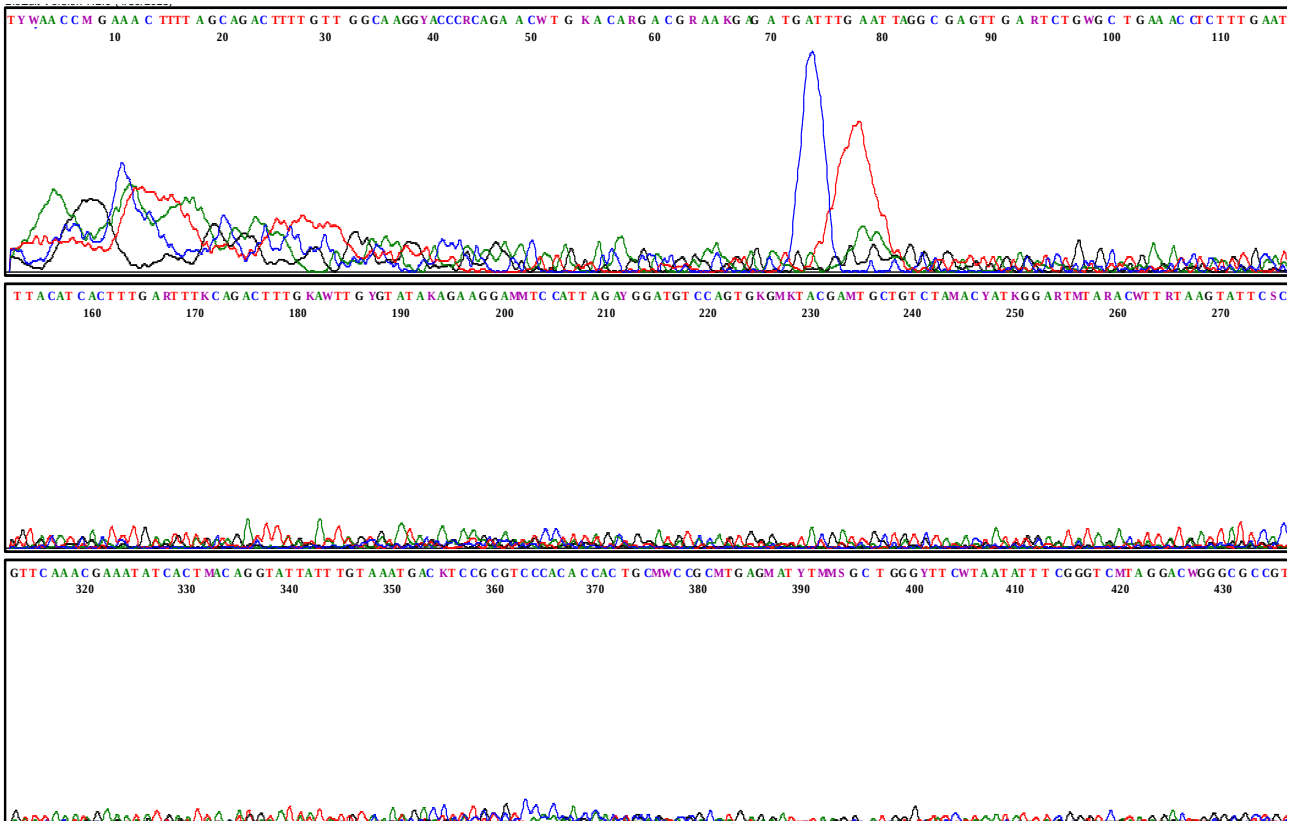
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Appendix E

Ethics clearance certificate

R14/49 Mr HP Earle et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150805

NAME: (Principal Investigator)	Mr HP Earle et al
DEPARTMENT:	Molecular Medicine and Haematology University of the Witwatersrand
PROJECT TITLE:	Molecular Diagnosis of High Risk Papilloma Virus Infection in a South African Patient Cohort
DATE CONSIDERED:	28/08/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr PM Durand
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	18/09/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/are are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature — Date: 18/09/2015

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

References

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