UNIVERSITY OF RWANDA

GENOTYPIC DISTRIBUTION OF HIGH-RISK HUMAN PAPILLOMAVIRUS INFECTIONS AMONG HIV-NEGATIVE WOMEN IN KIGALI-RWANDA: A CROSS-SECTIONAL STUDY USING AMPFIRE HPV GENOTYPE ASSAY

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By

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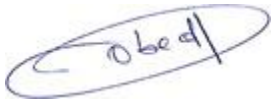
DECLARATION OF INDEPENDENT WORK

I, Obed TUYISHIME hereby declare that this research project submitted to the University of Rwanda, Rwanda for the degree Master of Science in Biotechnology is my own original work and has not been submitted before to any Institution by myself or any other person in fulfilment of the requirements to the award of any degree or any other qualification.

Obed TUYISHIME

Signature: 

Dates: July 27th, 2025

Main supervisor: Prof. Leon MUTESA

Signature:

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Leon Mutesa
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Dates: July 28th, 2025

DEDICATION

I dedicate this thesis project to:

My family for their unwavering love, encouragement, and support throughout my academic journey.

The Principal Investigator of the program for their guidance, inspiration, and continuous support.

The Government of Rwanda for its steadfast commitment to advancing education and research.

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LIST OF SYMBOLS AND ACCRONYMS

4vHPV: Quadrivalent HPV vaccine

9vHPV: Nonavalent HPV vaccine

AIDS: Acquired Immunodeficiency Syndrome.

CD4: Cluster of Differentiation 4

DNA: Deoxy-ribonucleic Acid.

GLOBOCAN: Global Cancer Observatory

HIV: Human Immunodeficiency Virus.

HPV: Human Papillomavirus.

hrHPV: High-risk Human Papillomavirus.

IARC: International Agency for Research on Cancer

IQR: Inter-quartile Range

LAMP: Loop-mediated Isothermal Amplification

LFB: Lateral Flow Biosensor

LMICs: Low- and Middle-Income Countries

MOH: Ministry of Health

PCR: Polymerase Chain Reaction

PLWH: People Living With HIV

RNEC: Rwanda National Ethics Committee

SSA: Sub-Saharan Africa

STIs: Sexual Transmitted Infections

USA: United States of America

VIA: Visual Inspection with Acetic acid

WHO: World Health Organisation

WLWH: Women Living With HIV

DEFINITION OF KEY TERMS

1. High-Risk Human Papillomavirus (hrHPV): A group of human papillomavirus genotypes that are strongly associated with the development of cancers of the cervix, anus, oropharynx, vagina, vulva, and penis. There are about 12 types of high-risk HPV, but HPV types 16 and 18 cause most HPV-related cancers. High-risk HPV is transmitted through intimate skin-to-skin contact, most often during vaginal, anal, or oral sex.

2. HPV Genotyping: The laboratory process of identifying specific types (genotypes) of HPV present in a sample. Genotyping helps distinguish between high-risk and low-risk types and is critical for assessing cancer risk and vaccine effectiveness.

3. AmpFire Assay: is an isothermal nucleic acid amplification assay for the qualitative genotyping of high-risk types of human papillomavirus (HPV). High risk HPV specific primers and fluorescent probes are used to amplify regions of viral genomic DNA including E6/E7 regions under isothermal conditions.

4. HIV-negative Women: Refers to women who have tested negative for the human immunodeficiency virus (HIV).

5. Cervical Cancer Screening: The process of detecting early changes in cervical cells that may lead to cancer. Current screening methods include Pap smears, visual inspection with acetic acid (VIA), and HPV DNA testing-especially for high-risk genotypes.

6. HPV Vaccine Effectiveness: The ability of HPV vaccines (e.g., bivalent, quadrivalent, nonavalent) to prevent infections caused by the HPV genotypes they target. Effectiveness can be measured through measurement of Antibody response and reduced prevalence of vaccine-covered HPV types and related cervical lesions in vaccinated populations.

7. Multiple HPV Infections: The presence of more than one HPV genotype in a single individual. Multiple infections are common, particularly in young women with high sexual exposure and may affect the risk of persistent infection and disease progression.

ABSTRACT

Background: Cervical cancer remains the leading cause of cancer-related morbidity and mortality among women in Rwanda, despite a successful rollout of a national human papillomavirus (HPV) vaccination program in 2011. Understanding the distribution of high-risk human papillomavirus (hrHPV) genotypes in the post-vaccination era is critical for improving screening and prevention strategies.

Objective: This study aimed to determine the prevalence and distribution of high-risk human papillomavirus genotypes and their association with HPV vaccination status among HIV-negative women in Kigali.

Methods: A cross-sectional study was conducted involving 216 cervicovaginal samples collected from HIV-negative women between 2021-2023. High-risk HPV genotyping was performed using the AmpFire multiplex isothermal amplification assay. Descriptive statistics, Chi-square and Fisher's exact tests were used to assess genotype-specific prevalence and associations between vaccination status and HPV positivity.

Results: The overall hrHPV prevalence was 35.2%, with 13.9% of participants showing multiple hrHPV infections. The prevalence of HPV-16 and HPV-18 was 2.3% and 1.9% respectively. Noncovered-vaccine hrHPV types, particularly HPV-39 (9.3%), HPV-52 (5.6%), and HPV-58 (5.6%), were most frequent. Vaccinated women had significantly lower hrHPV prevalence (29.3%) than unvaccinated (48.5%) ($p=0.007$). HPV-58 was more prevalent in unvaccinated individuals compared to the unvaccinated ($p = 0.032$), suggesting possible cross-protection for vaccinated women.

Conclusion: The study demonstrates a relatively high prevalence of noncovered-vaccine hrHPV types in HIV-negative women despite high national vaccine coverage. The findings highlight the need for broader vaccine formulations and support the integration of molecular screening tools like AmpFire assay into HPV surveillance in Rwanda.

Keywords: hrHPV, HPV genotyping, AmpFire, HPV Vaccine

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CHAPTER 1. INTRODUCTION

1.1. BACKGROUND

Cervical cancer is the fourth most common cancer among women globally, with an estimated 604,000 new cases and 342,000 deaths in 2020 (Sung et al., 2021); the burden is disproportionately high in low and middle-income countries (LMICs) , including those in sub-Saharan Africa(SSA), due to limited access to Human Papilloma Virus(HPV) screening, vaccination, and early treatment(Mukama et al., 2020).

In the WHO African Region, cervical cancer is the second most common cancer among women, (Ferlay et al., 2024). According to the International Agency for Research on Cancer(IARC) global cancer observatory report in 2022, cervical cancer is the leading cause of cancer-related deaths among women in Rwanda, with an estimated 866 new cases and 609 deaths reported annually(WHO/IARC, 2022). Although Rwanda has achieved over 90% HPV vaccination coverage among school-based girls since the introduction of national program in 2011(Murenzi & Mungo, 2023), the current vaccine's target only a limited range of high risk genotypes. As a result, it cannot be solely relied upon for complete protection against all oncogenic types. Therefore, it is essential to understand the dynamics of HPV infection to improve strategies for prevention and reduce the morbidity and mortality rate of cervical cancer in Rwanda.

Unlike many other cancers, cervical cancer has a well-established etiology-persistent infection with HPV and is highly treatable when detected early (Mukama et al., 2020). This makes it one of the few cancers for which timely and accurate screening and prognosis can significantly reduce morbidity and mortality. While certain low risk HPV types are associated benign lesions such as vaginal warts, high risk types, particularly HPV16 and HPV18 are responsible for approximately 70 % cervical cancer worldwide (Murenzi et al., 2024) . Consequently, most research and clinical screening programs have focused on HPV-16 and HPV-18. However, other hrHPV genotypes HPV-31,-33,-35,-45.-52,-58) are responsible for the remaining 25%-35% of cervical cancer cases (So et al., 2016) and contribute significantly to disease burden.

HPV infects basal epithelial cells of the anogenital tract through microabrasions in the mucosa. Once inside the basal layer, the virus establishes persistent infection by maintaining its genome as an episome and expressing early viral proteins (E1, E2, E6, and E7)(Tomaić, 2016). Among

these, E6 and E7 oncoproteins play a pivotal role in oncogenesis: E6 promotes degradation of the tumor suppressor p53, while E7 binds and inactivates the retinoblastoma protein (pRb), leading to uncontrolled cell proliferation and genomic instability (Moody & Laimins, 2010). Persistent hrHPV infection, particularly with HPV16 and 18, can result in integration of viral DNA into the host genome, further enhancing oncogene expression and driving progression from precancerous lesions (cervical intraepithelial neoplasia, CIN) to invasive carcinoma (Schiffman et al., 2016). Notably, while most HPV infections are transient and cleared by the immune system within 1-2 years, persistence of hrHPV is the key determinant of cervical cancer risk(Murenzi et al., 2024). HIV co-infection and immunosuppression also impair viral clearance, contributing to higher rates of persistence and progression among women living with HIV(Kelly et al., 2018).

Globally, only a third of girls reside in countries that have introduced the HPV vaccine, with LMICs the least likely to offer the vaccine (So et al., 2016). In Rwanda, the introduction of the HPV vaccine targeting HPV types (HPV6, 11, 16, and 18) has marked a major advancement in cervical cancer prevention. The program achieved over 90% coverage, particularly among schoolgirls who benefited from a national catch-up program. As a result, the prevalence of vaccine covered high risk HPV types, especially HPV-16 and HPV-18 has declined the prevalence of vaccine(Sayinzoga et al., 2023). However, cervical cancer screening that targets the HPV genotype is limited and more expensive in the country, and the most available method only target HPV-16 and HPV-18 genotypes among other hrHPV types. Thus, broader genotyping data and knowledge on the distribution of all hrHPV types are essential to inform comprehensive prevention strategies and support the integration of non-vaccine hrHPV types into national screening and surveillance efforts.

The current guidelines recommend directly referring women who tested positive for HPV16/18 for colposcopic evaluation while the non-16/18 hrHPV positive women would be triaged using cytology (Bai et al., 2023),(Wu et al., 2022). HPV testing has a lower specificity than cytology, and the management protocol for women who are negative for intraepithelial lesion or malignancy (NILM) but positive for non-16/18 hrHPVs remains a controversial issue in the current screening context (Wu et al., 2022). Furthermore, approximately 10% cervical high-grade lesions caused by non- 16/18 hrHPVs might be undetected, particularly in areas with

limited proficient cytologists (Bai et al., 2023), poor attendance rates, and loss to follow-up may be a relevant issue to those particular group of people.

In HIV-negative women, the immune system is generally more effective at clearing HPV infections within 1-2 years. Nevertheless, persistent infections with high-risk genotypes can still occur and lead to precancerous lesions or cancer if undetected at an early stage(Murenzi et al., 2024). A comprehensive review identified factors associated with increased risk of cervical or vaginal HPV infection among HIV-negative women, including sexual activity, a history of sexually transmitted infections (STIs) or genital warts, inconsistent condom use, hormonal contraception use, smoking, perimenopausal status, , and being withing the reproductive age range of 20-40 years (del Pino et al., 2024).

Despite Rwanda's successful implementation of a national HPV vaccination program in 2011 (Sayinzoga et al., 2023) and ongoing cervical cancer screening efforts, there remains a critical need to evaluate the efficacy of the vaccine and monitor the current distribution of high-risk HPV genotypes, particularly in vaccinated and unvaccinated populations. While vaccine-targeted genotypes like HPV-16 and -18 are expected to decline, emerging evidence suggests a possible increase in non-vaccine hrHPV types, potentially shifting the burden of cervical disease. Understanding the real-world impact of the vaccination program on genotype prevalence is essential. Additionally, exploring the feasibility of integrating reliable molecular testing tools, such as the AmpFire assay, into national screening protocols might enhance early detections and surveillance capacity. The findings underscore the crucial data to that will help to inform public health policy, improve screening strategies, and guide future HPV vaccine updates.

1.2. PROBLEM STATEMENT

Cervical cancer remains one of the leading causes of cancer-related morbidity and mortality among women globally, with the burden disproportionately affecting sub-Saharan Africa, including Rwanda (Ferlay et al., 2024; Sayinzoga et al., 2023). According to IARC/ global observatory report 2022, cervical cancer is the commonly diagnosed cancer among women in Rwanda(WHO/IARC, 2022). Persistent infection with hrHPV is a well-established etiological factor in the development of cervical cancer (Mukama et al., 2020). Although HPV16 and HPV18 genotypes account for the majority of 70% of all cases, there is increasing evidence that

highlights the significant contribution of other high-risk genotypes such as HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 that significantly contribute to disease burden, particularly in low resource settings (Murenzi et al., 2024).

Rwanda has made significant interventions in HPV prevention through vaccination and cervical cancer screening programs, particularly targeting HP-6, -11, -16, and -18 (Sayinzoga et al., 2023). However, existing epidemiological data remain limited to these vaccine-targeted genotypes and largely focused on women living with HIV, leaving a significant knowledge gap regarding hrHPV genotypes among HIV-negative women, that represent the majority of the female population. This gap hinders the development of comprehensive, population-wide prevention strategies. Additionally, currently available HPV screening methods in Rwanda often detect only HPV16 and HPV18 and remain costly and inaccessible for routine population-based screening. These limitations might hinder the effectiveness of national programs and delay progress toward WHO targets for cervical cancer elimination.

The lack of genotype-specific prevalence data among the female population compromises Rwanda's ability to tailor screening protocols, update vaccine policies and monitor vaccine impact. Without this information, public health interventions may fail to address the full spectrum of hrHPV infections, resulting in continued transmission, missed diagnoses and preventable disease.

The introduction of the AmpFire HPV genotyping assay presents an ideal alternative, capable of detecting 14 hrHPV genotypes simultaneously within one hour (Kanyabwisha et al., 2023). This study aims to characterize the genotypic distribution of hrHPV among HIV-negative women in Kigali using the AmpFire HPV Genotyping assay. The findings will provide critical data to support evidence-based national strategies for cervical cancer screening, surveillance and vaccine planning.

1.3. RESEARCH QUESTIONS

1. What is the overall prevalence of high-risk HPV infections among HIV-negative women in Kigali?
2. Which high-risk HPV genotypes are most detected among HIV-negative women in Kigali?

3. What is the association between HPV vaccination status and the prevalence of vaccine-covered (HPV-16/18) and non-covered high-risk HPV genotypes among HIV-negative women in Kigali?

1.4. STUDY OBJECTIVES

1.4.1. Main Objective

To determine the prevalence and distribution of high-risk human papillomavirus genotypes and their association with HPV vaccination status among HIV-negative women in Kigali.

1.4.2. Specific Objectives

1. To determine the overall prevalence of high-risk HPV infections among HIV-negative women in Kigali.
2. To determine genotype specific distribution of hrHPV genotypes in HIV-negative women in Kigali.
3. To evaluate the association between HPV vaccination and the prevalence of vaccine-covered and non-covered hrHPV genotypes among HIV-negative women in Kigali.

1.5. SIGNIFICANCE OF THE STUDY

This study provides a detailed genotypic profile of high-risk human papillomavirus (hrHPV) among HIV-negative women in Kigali-Rwanda using cervicovaginal swab specimens. By utilizing the AmpFire genotyping assay, it demonstrates the assay's applicability and effectiveness for HPV detection in the study population. The results provide valuable insights into the current distribution of both vaccine-covered and non-covered vaccine hrHPV types, underscoring the ongoing burden of non-16/18 genotypes. These findings have significant implications for national HPV surveillance and may influence policy modifications, such as the evaluation of broader vaccine formulations such as the 9vHPV vaccine. Additionally, the study supports the operational feasibility of implementing the AmpFire assay in large-scale screening efforts in resource-limited settings, aligning with Rwanda's ongoing cervical cancer elimination strategies.

CHAPTER 2. LITERATURE REVIEW

2.1. High risk Human Papillomavirus and cervical cancer

Human papillomavirus (HPV) is the most common sexually transmitted infection globally and is the primary etiology agent in the development of cervical cancer, particularly in low- and middle-income countries (LMICs) (Dunne & Park, 2013). In 2022, worldwide, cervical cancer was the fourth most common cancer in women, with around 660,000 new reported cases, where the majority 94% of the 350,000 deaths caused by cervical cancer occurred in low- and middle-income countries. Although women living with HIV represent only about 5% of global cervical cancer cases, they are approximately six times more likely to develop cervical cancer compared to HIV-negative women(WHO, 2020).

More than 200 HPV genotypes had been identified, with approximately 12 classified genotypes as high-risk (hrHPV) due to their oncogenic potential. These include HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58,and 59. Of these, HPV16 and HPV 18, are responsible for 70% of HPV-related cancers worldwide (WHO/IARC, 2022). Cervical cancer, primarily caused by persistent infection with high-risk HPV types, accounts for approximately 3.1% of all female cancers globally. However, the burden is disproportionately higher in low-income countries, where it comprises up to 7.7% of cancers, compared to around 2.2% in high-income countries(Ferlay et al., 2024).

In Rwanda, cervical cancer ranks as the second leading cause of female cancer and the first most common female cancer in women aged 15 to 44 years in Rwanda (Bruni et al., 2023). The country has made significant progress in cervical cancer prevention through National Cervical Cancer Prevention Program, which is integrated into the broader National Strategic Plan for Non-Communicable Diseases(MOH Rwanda, 2020). The Ministry of Health recommends screening women aged 30–49 using a combination of Visual Inspection with Acetic Acid (VIA), HPV DNA testing, and a screen and treat approach. Pilot studies evaluating tools like CareHPV, Hybrid capture assays have demonstrated feasibility and diagnostic sensitivity. Rwanda also aims to align with WHO's 90–70–90 strategy, aiming to achieve 90% HPV vaccination coverage, 70% screening coverage and 90% access to appropriate treatment for pre-cancer and cancer cases(MOH Rwanda, 2020).

2.2. Pathophysiology of Human papillomavirus

Human papillomavirus (HPV) initiates infection when small epithelial abrasions allow the virus to access the basal layer of the cervical epithelium. The virus establishes its genome as episomes and begins early gene expression, including E1, E2, E6, and E7(Tomaić, 2016). While E1 and E2 regulate viral replication and transcription, the viral oncogenes E6 and E7 are crucial for oncogenic transformation, as they disrupt host tumor suppressor proteins such as p53 and retinoblastoma protein (pRb)(Moody & Laimins, 2010). This interference leads to deregulated cell proliferation, impaired apoptosis, and accumulation of DNA damage.

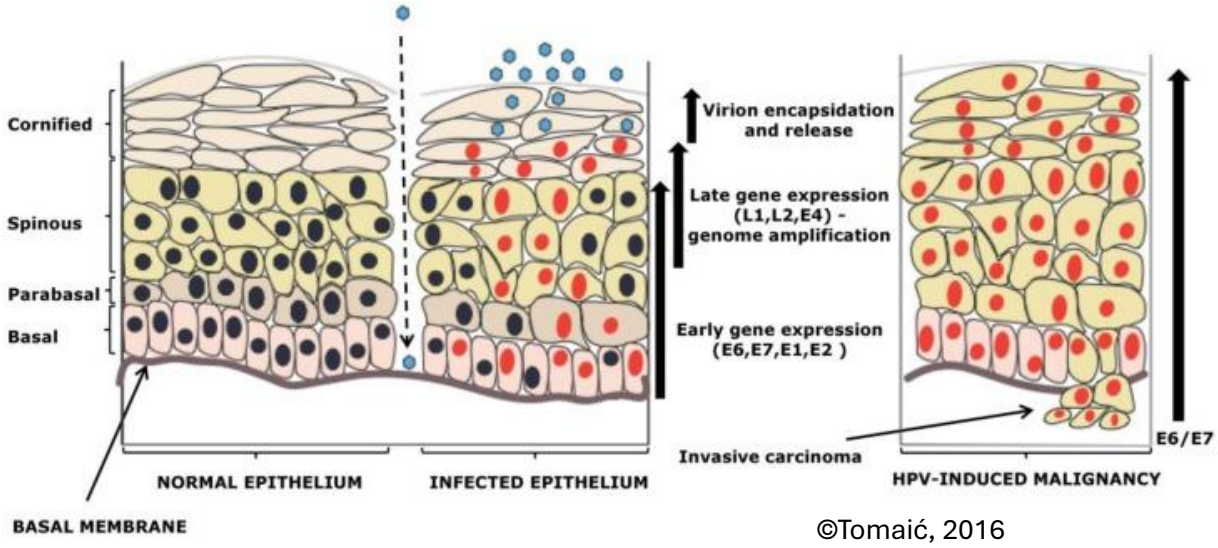

Figure 1: Productive HPV life cycle to cancer development

As infected cells migrate toward the epithelial surface, viral replication proceeds in synchrony with epithelial differentiation. Late genes (L1, L2) are expressed in the upper epithelial layers, leading to capsid assembly and virion release. In most women, the infection remains transient and is cleared by the immune system within one to two years. However, persistence of high-risk HPV (hrHPV) types is a critical determinant for progression to precancerous lesions and cervical cancer(Murenzi et al., 2024). Persistent expression of E6 and E7 disrupts genomic stability, promoting chromosomal abnormalities, telomerase activation, and resistance to apoptosis.

Over time, persistent hrHPV infection causes histological progression from cervical intraepithelial neoplasia (CIN) grades 1–3 to invasive cervical carcinoma. CIN1 represents mild dysplasia, usually associated with productive infection and high viral load, while CIN2 and

CIN3 reflect increased genomic instability, uncontrolled proliferation, and higher oncogene expression(Schiffman et al., 2016). When left unchecked, these lesions may breach the basement membrane, developing into invasive carcinoma. This malignant transformation is typically driven by integration of viral DNA into the host genome, resulting in dysregulated oncogene expression and loss of viral regulatory elements.

HPV-induced carcinogenesis is therefore a multistep process influenced by viral persistence, host immune responses, and cofactors such as HIV infection, smoking, and reproductive factors. HIV infection, in particular, accelerates HPV persistence and progression by weakening cell-mediated immunity, explaining the higher prevalence and severity of hrHPV-related disease in women living with HIV. In contrast, in HIV-negative women, persistent hrHPV remains the primary driver of cervical carcinogenesis, reinforcing the importance of preventive vaccination and molecular screening strategies.

2.3. High-Risk HPV Genotypes in Africa and Rwanda

Globally, HPV types 16 and 18 cause over 70% of cervical cancers, but other types like 31, 33, 45, 52, and 58 are increasingly detected, especially in African populations (Ogembo et al., 2015). African studies have shown a wide diversity of hrHPV genotypes, with types like HPV-52, -35, and -58 sometimes more prevalent than HPV-16 and -18 (Taku et al., 2021). The cervical cancer burden among low-and middle-income countries are mainly influenced by the inequalities in access to vaccines, screening and treatment services, high prevalence of HIV, social and economic determinants including sex, gender and poverty (Mukanyangezi et al., 2019).

A systematic review conducted by Seyoum and colleagues on the distribution of hrHPV in Africa have shown that, in Africa the hrHPV genotypes that mainly cause cervical cancer to vary from country to country due to the vicinity of the study and the health status of women participated in the study. For example, HPV-16 and -52 in South Africa and Tanzania, HPV-16, and -35 in Zimbabwe, and HPV-53 and -68 in Madagascar were the most common genotypes to cause cervical cancer among women. Similarly, studies conducted in East African countries have demonstrated a diverse distribution of high-risk HPV genotypes. In Uganda, HPV-52 and -58; in Tanzania, HPV-52 and−16; in Kenya, HPV-58 and−16; and in Ethiopia, HPV-16 and -52 genotypes were identified as the main causes of hrHPV infections (Seyoum et al., 2022). On the

other hand, he distribution of hrHPV genotypes in the West African countries has a different pattern from the southern and Eastern African countries. In Senegal, HPV-52 and−31, in Burkina Faso, HPV-52 and -59, and in Nigeria, HPV-35 and−16 genotypes were identified as the leading causes of the disease (Seyoum et al., 2022). These findings underscore the non-uniform distribution of the hrHPV genotypes across the continent and highlight the limitations of adopting a one-size-fits-all approach to HPV prevention. Genotype-specific variation suggests that national cervical cancer prevention strategies must be tailored to local epidemiological profiles.

In Rwanda, a study conducted by Mpunga and colleagues on the Human papillomavirus genotypes in cervical and other HPV-related anogenital cancer in Rwanda, HPV-16 was the predominant hrHPV type in cervical cancer (55.0%), followed by HPV-18 (16.6%) and HPV-45 (13.4%) (Mpunga et al., 2020). These findings align with global trends, where HPV-16 and HPV-18 account for most cervical cancer cases. However, the notable presence of HPV-45, which is not included in the quadrivalent vaccine, highlights the need for monitoring other oncogenic types beyond the traditional vaccine targets.

2.4. HPV Persistence in HIV-Positive vs. HIV-Negative Women in Rwanda

Persistent high-risk HPV (hrHPV) infection is a key risk factor for progression to cervical cancer. Evidence has consistently shown that women living with HIV (WLWH) have a higher risk of persistent hrHPV than their HIV-negative peers, largely due to immune suppression. In Rwanda, a longitudinal cohort study by Veldhuijzen and colleagues on high-risk HIV-negative women had found a hrHPV prevalence of 47%, cumulative incidence of 28%, and persistence of 32% over a mean follow-up of 17 months(Veldhuijzen et al., 2011). By comparison, among HIV-positive women in the same study, overall HPV prevalence reached 72.2%, indicating a substantially elevated burden(Veldhuijzen et al., 2011).

Similarly, Mukanyangezi et al. reported that more than half of baseline hrHPV infections persisted, and these persistent infections were strongly associated with cytological abnormalities(Mukanyangezi et al., 2019). Low CD4 counts and high HIV viral load were noted risk factors for reduced clearance. Another nested analysis using the AmpFire assay among screen-positive WLWH reported that 37% of initially detected hrHPV infections persisted, with type-specific persistence highest for HPV-16 (47.7%) and HPV-33 (56.7%), while HPV-39,

HPV-51, and HPV-66 had significantly lower persistence (6.7%, 15.6%, and 17.9%, respectively)(Murenzi et al., 2021). Lower CD4 was also associated with higher persistence rates.

In contrast, persistence of hrHPV was significantly lower or near zero among HIV-negative Rwandan women over a similar timeframe (Murenzi et al., 2021). The observed differences in HPV persistence by HIV status underscore the importance of tailored screening strategies and interventions in populations with HIV. These findings highlight stark differences in HPV persistence dynamics between HIV positive and HIV negative female populations. While the higher burden among WLWH has drawn research attention, there remains a paucity of genotype-specific data among HIV negative women. who comprise the majority of the population. Therefore, a deeper understanding of hrHPV molecular epidemiology in this underrepresented group is urgently needed to guide national cervical cancer prevention efforts.

2.5. HPV Vaccination and Effectiveness

Human papillomavirus (HPV) vaccination is a cornerstone in the global strategy to eliminate cervical cancer. The World Health Organization (WHO) recommends vaccinating girls aged 9–14 years before sexual debut, with the goal of achieving at least 90% coverage in this target population by 2030 as part of the cervical cancer elimination initiative (WHO, 2020).

Globally, the introduction of HPV vaccines particularly the bivalent (HPV-16/18), quadrivalent (HPV-6,-11,-16-18), and nonavalent (HPV-6,-11,-16,-18,-31,-33,-45,-52,-58) vaccines has led to significant reductions in vaccine-targeted type HPV infections, genital warts, and cervical precancerous lesions in vaccinated populations. For example, studies from countries with high coverage, such as Australia and the UK, have demonstrated up to an 83% reduction in HPV-16/18 infections within a decade of vaccine introduction(Drolet et al., 2019).

In Rwanda, the national HPV vaccination program was launched in 2011 using the quadrivalent HPV vaccine targeting types 6, 11, 16, and 18. The country achieved over 90% coverage through a school-based strategy, making it one of the first low-income countries to implement a national HPV vaccination program at such scale(Binagwaho et al., 2012). Evidence of vaccine effectiveness in Rwanda is emerging. A post-vaccination study by Sayinzoga and

colleagues(Sayinzoga et al., 2023) reported a significant decline in vaccine-type HPV prevalence (HPV-6, -11, -16, -18) from 12% pre-vaccination to 5% post-vaccination among young women. Furthermore, our study also showed a significantly lower hrHPV prevalence in vaccinated participants (29.3%) compared to unvaccinated ones (48.5%), $p = 0.007$. Reductions in non-vaccine α-9 HPV types (HPV-31, -33, -35, -52, -58) have also been observed, suggesting cross-protection from the quadrivalent vaccine. Baussano and colleague reported a 58% reduction in these types in Rwanda and 63% in Bhutan, both of which had maintained high vaccine coverage(Baussano et al., 2020).

Despite this progress, non-16/18 high-risk HPV types remain prevalent. This highlights the need for expanded vaccine formulations such as the nonavalent vaccine (9vHPV vaccine) and ongoing cervical cancer screening, especially among unvaccinated or partially vaccinated populations.

2.6. HPV Detection Methods: AmpFire, and Other Molecular Assays

Molecular detection method of HPV DNA using polymerase chain reaction (PCR)is the gold standard for HPV genotyping, offering high sensitivity and specificity. Traditional assays like Roche Linear Array, Cobas 4800, and Hybrid Capture 2 are widely used in clinical and research (Arbyn et al., 2021). These platforms are well validated but typically require specialized equipment, multi-step DNA extraction and longer processing times, which may limit feasibility in resource-constrained environments like Rwanda.

In contrast isothermal amplification techniques, like the AmpFire HPV Genotyping Assay of Atila Biosystems, offer faster, multiplex detection of 14 hrHPV genotypes without DNA extraction, making them ideal for low-resource settings (Kanyabwisha et al., 2023). Moreover, an evolving ultrasensitive and specific Cas12a-based biosensor has been developed for the simple detection of HPV16 and HPV18 (Mukama et al., 2020). This method, termed “CIALFB,” combines CRISPR-Cas12a trans-cleavage activity, loop-mediated isothermal amplification (LAMP), and a lateral flow biosensor (LFB)(Mukama et al., 2020). This approach provides a simple and rapid alternative to traditional methods, particularly suitable for decentralization or point of care application in low and middle-income countries. However, the method still requires validation, and its current ability to detect only HPV16 and HPV18 limits its generalizability as

an ideal screening tool. A study conducted by Kaleigh and colleagues demonstrated AmpFire assay significantly reduced processing time (90 minutes vs 270 minutes) and equipment dependency compared to Roche Linear Array(Connors et al., 2021).

Given the need for scalable, cost-effective and genotype-inclusive screening technologies in Rwanda, AmpFire assay offers a practical and efficient alternative to traditional PCR platforms. Its ability to detect a broader range of hrPV genotypes quickly and with minimal inputs aligns well with the operational demands of community-level screening programs. Therefore, this study leverages the AmpFire assay to address the existing gap in genotype-specific hrHPV prevalence data, particularly among HIV negative women who are currently underrepresented in national HPV surveillance efforts.

CHAPTER 3. METHODOLOGY

3.1. Study Design

A laboratory-based analytical cross-sectional study nested within an ongoing mother U-54 project entitled “Long- term human papillomavirus vaccination effectiveness and immunity in Rwandan women living with and without HIV” was used detect the hrHPV from cervicovaginal swab specimen collected from HIV-negative women enrolled within U-54 project.

3.2. Study area and Population

This study involved women enrolled at five sites in Kigali: Rwanda Military Referral and Teaching Hospital, Gikondo Health Centre, WE-ACTx Private Clinic, Cor-Unum Health Centre, and Kacyiru Health Centre. Cervicovaginal samples were originally collected from these participants as part of the U-54 project and stored under appropriate conditions in the biobank at the Rwanda Military Referral and Teaching Hospital research laboratory, managed by Research for Development through the Einstein-Rwanda Research and Capacity Building Program. The confirmed HIV-negative women aged 18 years and above, whose stored cervicovaginal swab samples were available in good condition, were selected for hrHPV genotyping laboratory analysis.

3.3. Inclusion Criteria

- HIV-negative women
- Aged ≥18 years
- Availability of stored cervicovaginal swab samples

3.4. Exclusion Criteria

- HIV-infected Rwandan women
- Women with incomplete demographic data
- Insufficient sample volume
- Sample with poor quality

3.5. Sample Size

A total of 216 met the criteria out of 246 cervicovaginal swab samples, which were needed for the entire study and were tested for further laboratory analysis. An estimated sample size was adopted from a previous study titled *"Human papillomavirus infection in Rwanda at the moment of implementation of a national HPV vaccination programme"* reported that the overall prevalence of hrHPV among HIV-negative Rwandan women was 20% (Ngabo et al., 2016). The Fisher's formula was used to calculate the estimated sample size(Arya et al., 2012).

$$n = \frac{Z^2 p(1-p)}{e^2}$$

Where:

- **n=estimated total number of samples required**
- **Z=1.96 (for a 95% confidence level)**
- **P=0.2 (estimated prevalence)**
- **e=0.05 (desired margin of error)**

$$\boldsymbol{n = \frac{1.96^2 \times 0.2(1-0.2)}{0.05^2} = \frac{3.8416 \times 0.2 \times 0.8}{0.0025} \approx 246}$$

3.6. Sample processing and hrHPV DNA Detection Using the AmpFire Assay

The stored cervical vaginal swab samples were thawed at room temperature and mixed gently before subsequent analysis. The analysis was performed by following the AmpFire HPV Genotyping Assay protocol (Atila Biosystems, Mercury Drive, Sunnyvale, CA, USA) which allows simultaneous detection of 15 hrHPV genotypes: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, and -68. High risk HPV specific primers and fluorescent probes are used to amplify regions of viral genomic DNA including E6/E7 regions under isothermal conditions. The AmpFire assay is fast and highly sensitive method capable of multiplexing all 15 hrHPV genotypes in one run, and its cost-effectiveness makes it ideal for large-scale studies, especially in resource-limited settings.

The AmpFire HPV genotyping assay is an isothermal nucleic acid amplification method that does not require DNA extraction. It amplifies target HPV DNA directly from the sample using

a proprietary mix and identifies specific genotypes using fluorescent-labelled probes within 1 hour(Atila BioSystems, 2025).

Steps:

1. Dry cervicovaginal swabs were mixed with 2ml of 1X lysis buffer provided in the kit and incubated for 20min at 95 ^{o}C, and then the extract is ready for further PCR analysis.
2. The master mixes were prepared using the provided protocol. The assay is a multiplex isothermal amplification real-time system requiring four different master mixes, each designed to detect four specific HPV genotypes along with an internal control(β-globulin) in each reaction.
3. 5µl of sample mixture(extract) was then added directly to the reaction PCR master mix.
4. The resulting four reaction tubes for every sample were incubated in the PowerGene 9600 fluorescence real-time polymerase chain reaction (PCR) system at 60^{o}C with fluorescence from FAM/HEX/ROX/CY5 channels measured every minute.
5. Result interpretation, after running for approximately one hour. This experiment run was valid if the negative control showed no exponential curve and the positive control showed exponential curves. The next step was to examine the set of four tubes corresponding to a specimen. Multiplex HPV infections could result in multiple exponential curves for a specimen. If no exponential curve other than internal control (Hex channel in PM-3 tube) is present for a sample, this sample was considered negative. If there was no exponential amplification curve in any of the four tubes or any fluorescence channels, the sample would have failed the test. A failed sample usually indicates that there is not enough DNA in the sample, or sample processing was not accurate, and it was then repeated.

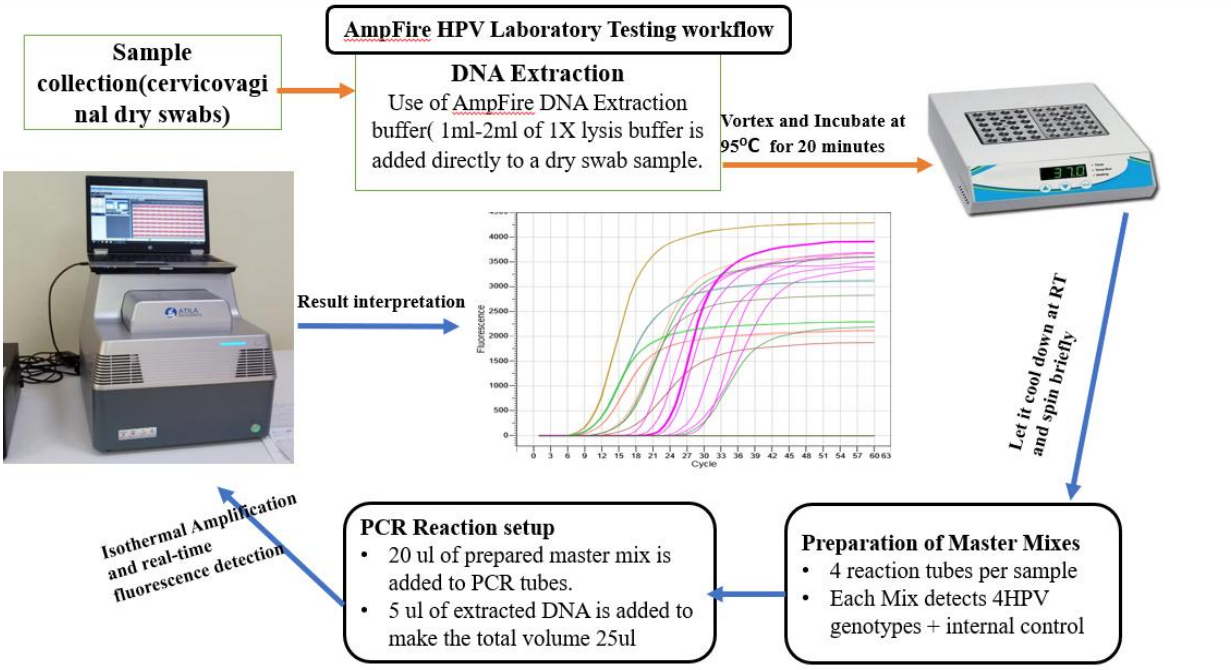

Figure 2: AmpFire HPV genotyping Assay Laboratory workflow

3.7. Data Analysis

The data were entered and cleaned using Microsoft Excel (Microsoft Corp, Santa Rosa, CA, USA) and then exported to IBM SPSS Statistical Developer version 25 for statistical analysis. Descriptive statistics, including frequencies and percentages, were used to summarize the prevalence of individual HPV genotypes and the frequency of single and multiple infections. Associations between hrHPV positivity and variables such as age, vaccination status were assessed using Pearson's chi-square test and Fisher's exact test. A p-value of < 0.05 was considered statistically significant.

3.8. Ethical considerations

The ethical approval was used under the U54 project with the Rwanda National Ethics Committee approval number: RNEC520/2024. The permission to use the samples was approved from project PIs. Moreover, the participants' confidentiality was guaranteed by using codes and accessed by authorized personnel only.

CHAPTER 4. RESULTS

This study analyzed cervicovaginal swab samples from 216 HIV-negative women enrolled at five health facilities in Kigali, Rwanda. The median age of participants was 26 years (IQR: 23–29). Nearly half of the women (49.5%) were aged 25–30 years, while 69.4% reported receiving the HPV vaccine through the national vaccination program.

Overall, the prevalence of high-risk HPV (hrHPV) was 35.2%, with 13.9% of participants showing multiple hrHPV infections. Among the 15 hrHPV genotypes detected, HPV-39 (9.3%), HPV-52 (5.6%), and HPV-58 (5.6%) were the most prevalent, whereas HPV-16 (2.3%) and HPV-18 (1.9%) were detected at relatively lower rates. Importantly, hrHPV positivity was significantly lower among vaccinated women (29.3%) compared to unvaccinated women (48.5%) ($p = 0.007$).

The following subsections present the detailed demographic characteristics, overall hrHPV prevalence, and genotype-specific distribution, as well as associations between vaccination status and hrHPV infections.

***Table 1*: Demographic characteristics of HIV negative Rwandan Women included in the study(n=216)**

Variable	n	%
Age, years, Median age=26(IQR: 23–29)		
<20	14	6.5
20-24	67	31.0
25-30	107	49.5
>30	28	13.0
HPV_Vaccination status*		
Vaccinated	150	69.4
Unvaccinated	66	30.6

**Self-reported vaccination status*

As shown in Table 1: A total of 216 women were included in the analysis, stratified by age and vaccination status. The median age of the participant is 26 years old (interquartile range (IQR: 23-29), and most participants clustered between 25 and 30 years old. Among the total participants, 150 (69.4%) reported having received the HPV vaccine, while 66 (30.6%) were unvaccinated.

Table 2: Prevalence of HPV and type-specific genotypic distribution among HIV-negative Women (N = 216)

Overall prevalence	**n**	**%**
Overall positivity	76	35.2
Multiple_HPV types	30	13.9
HPV16 and others	2	0.9
HPV18 and others	3	1.4
Other_hrHPV genotypes	67	31.0
Overall type-Specific hrHPV genotypes		
16	5	2.3
18	4	1.9
31	4	1.9
33	4	1.9
35	8	3.7
39	20	9.3
45	8	3.7
51	9	4.2
52	12	5.6
53*	11	5.1
56	8	3.7
58	12	5.6
59	5	2.3
66	7	3.2
68	5	2.3

**Possibly high-risk*

This table 2, combine the study objective 1 and 2; The overall prevalence of high-risk human papillomavirus positivity was 35.2% (n=76). Of these, 31% of participants tested positive for hrHPV genotypes not covered by the quadrivalent vaccine, underscores a possibility of shift in genotype dominance in the post-vaccination era. Moreover, 13.9% multiple hrHPV infections were detected.

Most notably, HPV-39 emerged as the most prevalent genotype detected in 20(9.3%), followed by HPV-52 (5.6%) and HPV-58 (5.6%). In contrast vaccine-covered types of HPV-16 and HPV-18 was low compared to other HPV genotypes with 2.3% and 1.9% respectively. These findings may reflect the impact of the national HPV vaccination program, whole also suggesting a potential genotype shift toward non vaccine hrHPV types in this HIV negative woman.

Table 3: Association between HPV vaccination status and prevalence of vaccine-covered and non-covered hrHPV among study population(N=216)

Variable	Vaccination Status	hrHPV Negative (n, %)	hrHPV Positive (n, %)	Chi-Square	p-value*
Vaccination status	Vaccinated	106(70.7%)	44(29.3%)	7.371	**0.007**
	Unvaccinated	34(51.5%)	32(48.5%)		
HPV-16	Vaccinated	146 (97.3%)	4 (2.7%)	0.269	0.604
	Unvaccinated	65 (98.5%)	1 (1.5%)		
HPV-18	Vaccinated	147 (98.0%)	3 (2.0%)	0.059	0.808
	Unvaccinated	65 (98.5%)	1 (1.5%)		
Other hrHPV Types	Vaccinated	113 (75.3%)	37 (24.7%)	9.257	**0.002**
	Unvaccinated	36 (54.5%)	30 (45.5%)		
HPV-58	Vaccinated	145 (96.7%)	5 (3.3%)	4.62	**0.032**
	Unvaccinated	59 (89.4%)	7 (10.6%)		

**The statistical significance was set at p-value ≤0.05*

Overall hrHPV positivity was significantly lower in vaccinated individuals (29.3%) compared to unvaccinated (48.5%) (*p = 0.007)* suggesting a protective effect of the vaccination. HPV-16 and HPV-18, which are targeted by the current vaccine, showed no significant difference in infection rates between vaccinated and unvaccinated individuals (p= 0.604 and 0.808, respectively).

Other high-risk HPV types (non-16/18) were significantly more common in unvaccinated women (45.5%) than vaccinated ones (24.7%) (*p = 0.002*, indicating the importance of broader vaccine coverage). HPV-58, was also significantly higher in unvaccinated individuals than in vaccinated ones (10.6% vs 3.3%, p = 0.032), suggesting the genotype shift and cross-protection.

CHAPTER 5. DISCUSSION

This study aimed to determine the prevalence and distribution of high-risk HPV genotypes and their association with vaccination status among HIV-negative women in Kigali. The findings of this study underscore the distribution of the hrHPVs especially the non-vaccine targeted genotypes infection among the women in the study population, most of whom belong to the cohort that received the HPV vaccine following the introduction of Rwanda's national vaccination program in 2011.

The overall hrHPV prevalence among HIV-negative women in this study was 35.2%, with 13.9% exhibiting multiple hrHPV infections. Importantly, hrHPV positivity was significantly lower among vaccinated individuals (29.3%) compared to their unvaccinated counterparts (48.5%), ($p = 0.007$), highlighting the vaccine's effectiveness in reducing hHPV burden. These findings align with earlier report over time from Rwanda demonstrating high vaccine uptake and decreasing prevalence of vaccine-covered types over time (Murenzi & Mungo, 2023; Sayinzoga et al., 2023).

This study prevalence is slightly higher than the 31.8% hrHPV prevalence reported by Veldhuijzen and colleagues in 2011 among HIV-negative women in Kigali, and substantially higher than 8.2 % reported by Sinayobye and colleagues among HIV-infected and negative Rwandan women(Sinayobye et al., 2014; Veldhuijzen et al., 2011). Conversely, our results are lower than the 45.5% prevalence reported by Luchters and colleagues among female sex workers in Kenya, a population(Luchters et al., 2010) . These differences likely reflect a combination of factors, including variations in study populations and associated risk profiles, the timing and coverage of vaccination programs, and importantly, differences in HPV detection methods. Notably, Veldhuijzen and colleagues utilized the GP5+/6+ PCR assay, while this method has been widely applied in epidemiological studies and provides robust detection of a broad spectrum of HPV genotypes, it requires a conventional PCR setup, followed by hybridization or sequencing, which is more time-consuming and resource intensive. Sinayobye and colleagues employed the careHPV DNA test, which detects pooled high-risk HPV types without distinguishing individual genotypes, AmpFire allows rapid, 15 genotype-specific detection, while careHPV DNA test is suitable for high-throughput screening in low-resource settings, its inability to identify individual genotypes limits detailed epidemiological analyses

and assessment type-specific hrHPV distribution across different population(Sinayobye et al., 2014). Luchters and colleagues used TaqMan®-based real-time PCR assay which is considered the gold standard in molecular diagnostics due to its accuracy and quantitative capabilities, it is time consuming, requires more expensive equipment and technical expertise, limiting its use in some resource-limited laboratory settings. While our study used the AmpFire assay which offers a balance between rapid turnaround, cost-effectiveness, and genotype specificity, making it well-suited for low resource settings (Luchters et al., 2010).

The most frequent high-risk HPV genotypes identified in this study were HPV-39 (9.3%), HPV-52 (5.6%), and HPV-58 (5.6%) among our study population. These findings are in accordance with regional findings but diverge in order of predominance. Veldhuijzen and colleagues reported a high prevalence of HPV-52 and HPV-58 in both women living with and without in Rwanda (Veldhuijzen et al., 2011). The consistent detection of HPV-52 and HPV-58 across studies suggests these types to be highly prevalent in this setting, regardless of HIV status. However, HPV-39 being the most common genotype in this study differs from earlier findings, possibly due to differences in assay sensitivity (AmpFire), population characteristics, vaccine coverage and herd immunity. The persistent circulation of genotypes such as HPV-39, HPV-52, and HPV-58 highlights the need for continued monitoring of genotype distribution and the potential benefits of next-generation multivalent HPV vaccines that cover a broader range of hrHPV types.

HPV-16 and HPV-18, both targeted by the quadrivalent HPV vaccine (4vHPV) administered in Rwanda were found in 2.3% and 1.9% of participants, respectively. These prevalence rates showed no statistically significant differences between vaccinated and unvaccinated individuals ($p = 0.604$ for HPV-16, $p = 0.808$ for HPV-18), likely due to the small number of participants testing positive for these genotypes or possibly reflecting strong vaccine-induced immunity and herd protection. The vaccination program in Rwanda have improved the vaccine targeted genotype prevalence with substantially lower rates than those reported in pre-vaccination or among high-risk population studies. For instance, Luchters and colleagues reported HPV-16 and HPV-18 prevalences of 11.1% and 6.8% among female sex workers in Kenya high-risk population (Luchters et al., 2010). Similarly, Veldhuijzen and colleagues observed 5.4% and 3.2% prevalence for HPV-16 and -18, respectively among HIV-negative high-risk women in

Kigali before vaccine rollout(Veldhuijzen et al., 2011). This is also supported by study conducted by Sayinzoga and colleagues who reported a significant drop in the prevalence of vaccine-targeted types (HPV-6, -11, -16, -18) from 12% to 5% in post-vaccination cohorts in Rwanda, further supporting our findings and demonstrating the vaccine's effectiveness (Sayinzoga et al., 2023).

In our study, other high-risk HPV types not included in the 4vHPV vaccine administered in Rwanda were more prevalent (31%) compared to HPV-16 and -18 combined. These non-16/18 hrHPV types were significantly more common in unvaccinated women (45.5%) than in vaccinated women (24.7%) ($p = 0.002$). The current finding support results from a study conducted by Ashry and colleagues who reported an overall prevalence of hrHPV in 71% and 55.6% for other hrHPV types among HIV-negative women, suggesting an ongoing burden of non-vaccine HPV genotypes (Ashry et al., 2024).

Interestingly, HPV-58 was the only genotype showing a statistically significant association with vaccination status, being more common in unvaccinated women (10.6%) than vaccinated ones (3.3%) ($p = 0.032$). Although not directly targeted by the 4vHPV vaccine, this result may reflect partial cross-protection. Indeed, Sayinzoga and colleagues; Baussano and colleagues also reported significant reductions in other α-9 HPV types in post-vaccination (e.g., HPV-31, -33, -35, -52, and -58) (Baussano et al., 2020; Sayinzoga et al., 2023) supporting the existence of cross-protection.

CHAPTER 6. CONCLUSION AND RECOMMENDATION

This study's findings support the continuation and expansion of Rwanda's national HPV vaccination program, with a particular emphasis on the vaccine's effectiveness in reducing the prevalence of HPV-16 and HPV-18 among HIV-negative women. However, the substantial burden of non-vaccine targeted high-risk HPV types of especially HPV-39, HPV-52, and HPV-58, which were more prevalent than vaccine-targeted types, underscores the need to consider introducing broader-spectrum HPV vaccines such as the nonvalent vaccine.

Additionally, given the significant differences in hrHPV prevalence between vaccinated and unvaccinated groups, scaling up vaccination efforts to improve coverage among missed or unvaccinated cohorts is strongly recommended. The use of sensitive diagnostic platforms like AmpFire should also be evaluated for integration into national screening programs, particularly due to its capability to detect a wide range of hrHPV genotypes efficiently. Finaly, this study is limited to HIV-negative women in Kigali, which may not represent the broader Rwandan population. A broader, longitudinal studies are needed to evaluate nationwide HPV trends and vaccine impact more comprehensively.

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