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MOLECULAR DETECTION AND SUBTYPING OF CIRCULATING INFLUENZA VIRUS STRAINS IN RWANDA

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MOLECULAR DETECTION AND SUBTYPING OF CIRCULATING INFLUENZA VIRUS STRAINS IN RWANDA

BY

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Dissertation submitted in fulfillment of the requirements for the degree:

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Mr. Emmanuel KABALISA

Kigali-Rwanda, 2025.

DECLARATION

I, Liliane INGABIRE IYAMUREMYE hereby declare that this research project submitted to the University of 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.

Liliane INGABIRE IYAMUREMYE

Signature

DEDICATION

To the Almighty God;

To my supervisors;

To all my family;

To all my Friends;

I dedicate this work.

ACKNOWLEDGMENT

I am profoundly grateful and extend my deepest appreciation to the Almighty God for His mercy, blessings, and guidance, through whom this work has been made possible.

I sincerely acknowledge with gratitude the invaluable contributions of individuals, lecturers, friends, and family who have supported me throughout this journey. I would like to express my heartfelt appreciation to Dr. Jeanne Primitive UYISENGA, Dr. Jean Paul NSHIZIRUNGU, and Mr. Emmanuel KABALISA for their continuous guidance, encouragement, and technical support in the completion of this project.

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To all who contributed in one way or another, may God bless you abundantly.

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LIST OF ACRONYMS AND ABBREVIATIONS

PCR: Polymerase Chain Reaction

RRT PCR: Reverse Real Time PCR

GISRS: Global Influenza Surveillance and Response System

WHO: World Health Organization

CHUB: Centre Hospitalier Universitaire de Butare

CHUK: Centre Hospitalier Universitaire de Kigali

SARI: Severe Acute Respiratory Infection

ILI: Influenza Like Illness

pdm09: pandemic H1N1 2009

RNA: Ribonucleic Acid

HA: Hemagglutinin

NA: Neuraminidase

vRNPs: Viral Ribonucleoproteins

vRNA: Viral Ribonucleic Acid

cDNA: Complementary DNA

RSC: Rapid Sample Concentrator

NTC: Negative Template Controls

SC2: Sars Cov2

HEF: Hemagglutinin–esterase fusion

HPIV: Highly Pathogenic Influenza Virus

HIV: Human Immunodeficiency Virus

RNAi: Ribonucleic Acid interference

NPIs: Non-Pharmaceutical Interventions

ABSTRACT

Background: Influenza virus remains a significant global health threat, causing seasonal epidemics and occasional pandemics. Nearly 10% of the world's population is affected by influenza annually, with about half a million deaths each year. Rwanda, has established a national influenza surveillance system, where a lot of data is generated on the epidemiology and seasonality of influenza. There is limited recent molecular data on circulating influenza subtypes.

Objectives: This study aimed to identify the circulating influenza virus subtypes using real-time reverse transcription polymerase chain reaction (RT-PCR) among patients presenting with influenza-like illness (ILI) in selected health facilities in Rwanda.

Methodology: Nasopharyngeal samples were analyzed for influenza and later subtyped into A(H1N1) pdm09, A(H3N2), and other relevant subtypes. Demographic and epidemiological data were analyzed to assess the patterns of distribution by age groups and regions.

Results: This study on the molecular detection and subtyping of influenza in Rwanda found a 10% prevalence of influenza in 320 study participants, with the H1N1pdm09 subtype detected in 32 samples. Females had a higher infection rate (13.3%) compared to males (6.5%), with a significant association ($p = 0.044$). No significant correlation was found between age and H1N1pdm09 positivity ($p = 0.837$). Geographically, 62.5% of the cases were detected in eastern province indicating regional variation, Results provide essential, up-to-date information on influenza subtype distribution and help the national surveillance system through guidance of vaccine and outbreak preparedness strategies. The study serves as a critical stepping stone toward the implementation of influenza surveillance in Rwanda.

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CHAPTER 1. INTRODUCTION

1.1Background

Influenza viruses 'infections are among the leading causes of morbidity and mortality worldwide. It affects nearly 10% of the world's population with an estimated 290,000 to 650,000 respiratory deaths per year worldwide , (Singh et al., 2020) impacting low- and middle-income regions, including Africa . Influenza continues to be a pressing global health concern, responsible for recurrent seasonal outbreaks and occasional pandemics. Its impact is especially pronounced in low- and middle-income regions, including sub-Saharan Africa, where it contributes substantially to illness and death,(Ng & Gordon, 2015) despite considerable progress and efforts in national surveillance system, influenza related respiratory illness in Rwanda remain a public health concern necessitating continuous surveillance and evaluation of intervention strategies. (Nyamusore et al., 2018)

Populations such as pregnant women, the elderly, young children, and individuals with weakened immune defenses are particularly vulnerable to developing severe disease from respiratory viruses, including influenza(World Health Organization, 2011) These high-risks groups are more likely to develop complications, require hospitalization, or even face influenza-related death.(Talla Nzussouo et al., 2017)The burden of influenza related illness and death are influenced by several factors, including the predominant viral subtype, presence of comorbidities, delays in diagnosis, co-infections, and low vaccination coverage.(Africa, 2018)For instance, Subtypes like A(H3N2) are often linked to more severe illness in older adults, while A(H1N1) pdm09 is known to cause severe illness in younger individuals and pregnant women.(Oboho et al., 2016)

Of all the infectious diseases, influenza deserves the particular attention as it undergoes a high rate of antigenic change giving rise to a new type of influenza strain for which there is no immunity in the population, The virus is known for its high mutation rate and the capacity to undergo genetic reassortment, leading to the emergence of new strains that can evade existing immunity. (Petrova & Russell, 2018)

Globally, molecular surveillance of influenza through tools like RT-PCR and sequencing is well-established in high-income countries and supported by initiatives such as WHO's Global Influenza

Surveillance and Response System (GISRS). Since 2009-2010, a notable progress in laboratory capacity has been made in WHO African region, despite this improvement countries In Africa continue to face gaps in molecular surveillance although sentinel surveillance exists, molecular characterization is inconsistent, and many countries struggle to detect subtype shifts or reassortment events with is a significant limitation on epidemiological data, risk factors and disease burden.(WHO, 2015) In Rwanda, the influenza surveillance system has provided epidemiological data since 2008,(Nyatanyi et al., 2012) Available reports primarily focus on the detection of influenza-like illness (ILI) and have provided minimal insights into the specific demographic factors that may influence susceptibility to influenza infection in Rwanda, vulnerable groups such as children under five, pregnant women, the elderly, individuals with chronic illnesses remain underrepresented in existing data. Therefore, continuously monitoring the emergence and spread of circulating influenza subtypes is essential as it should be part of ongoing influenza surveillance. This study aimed to identify circulating influenza virus strains in Rwanda, generating evidence that directly support national public health preparedness and surveillance.

1.2 Problem statement

In Rwanda, surveillance between 2012 and 2014 estimated an annual average of 3,663 influenza-associated SARI hospitalizations (34.3 per 100,000 population), with children under five accounting for 72% of cases(Nyamusore et al., 2018)

Although Rwanda's sentinel surveillance system has provided valuable data on the overall burden of influenza-like illness (ILI) and SARI, the country lacks recent molecular data on circulating influenza virus subtypes. Without up-to-date information on subtype distribution, it is difficult to detect changes in circulating strains, select appropriate vaccine components, or prepare effectively for seasonal outbreaks.

This study aimed to address this gap by determining the prevalence and subtype distribution of influenza viruses circulating in Rwanda using real-time reverse transcription polymerase chain reaction (RT-PCR). By generating updated, subtype-specific data, the study strengthens Rwanda's influenza surveillance system and provide evidence to support more responsive public health interventions, including vaccine policy and outbreak preparedness.

1.3 Research objectives

1.3.1 General objective

To identify circulating subtypes influenza strains in Rwanda using RT-PCR.

1.3.2 Specific objectives:

1. To determine the prevalence of influenza virus infection collected from patients in Rwanda.
2. To detect the predominant influenza virus types and subtypes circulating in Rwanda.
3. To analyze the epidemiological and demographic patterns associated with each detected subtype

1.4 Research question

- What is the overall prevalence of influenza virus infection among patients presenting with influenza-like illness in Rwanda?
- Which influenza virus types and subtypes are most commonly circulating in Rwanda?

1.5 Significance of the study

This research carries important public health value as it addresses a key gap in Rwanda's surveillance system—namely, the lack of recent molecular evidence on circulating influenza subtypes. By providing updated information on the prevalence and distribution of these viruses, the study will strengthen the country's capacity to track influenza evolution, detect emerging strains, and mount timely responses to seasonal outbreaks. In addition, the findings will support the prioritization of high-risk groups and guide national vaccination strategies, ultimately contributing to a reduction in influenza-related illness, mortality, and the overall strain on Rwanda's healthcare system.

CHAPTER2: LITERATURE REVIEW

This chapter provides a review of existing literature on molecular genotyping of influenza viruses, emphasizing an overview of the virus itself, the prevalence of influenza infections in Africa, the distribution of circulating types and subtypes, epidemiological and demographic trends linked to these subtypes, as well as the current state and challenges of molecular surveillance.

2.1 Overview of Influenza Virus

2.1.1Morphology and structure of influenza

Influenza viruses are enveloped, single stranded RNA viruses belonging to the family of Orthomyxoviridae categorized as type A, B, C and D. The virus belongs to the genera *Alphainfluenzavirus*, *Betainfluenzavirus*, *Gammainfluenzavirus* and *Deltainfluenzavirus* , with a segmented genome of eight genes coding for up to 11 proteins including surface glycoproteins of HA(Hemagglutinin) and NA(Neuraminidase).(Javanian et al., 2021)

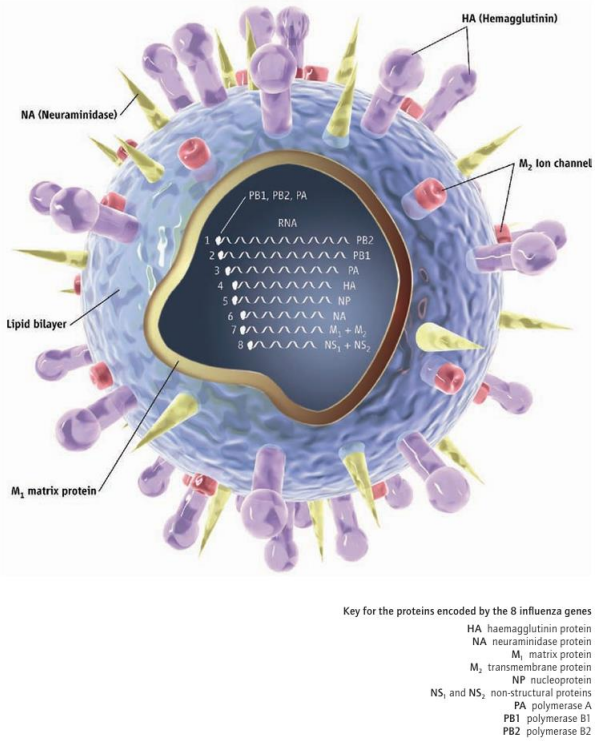


Figure 1 Influenza virus structure(World Health Organization, 2011)

2.1.2 Influenza Virus types and Subtypes

Influenza A viruses are classified into subtypes based on antigenic variation of their surface glycoproteins (NA and HA). Currently, there are 18 recognized subtypes of HA(H1-H18) so far, and 11(N1-N11) NA subtypes. With H1N1 and H3N2 representing the most prevalent circulating strains(Wu & Wilson, 2020)

In contrast to influenza A viruses, type B viruses are not divided into subtypes. They are classified into two lineages Victoria and Yamagata.(Javanian et al., 2021)

During infection the influenza virus type A and B attacks the host cell, the HA(Hemagglutinin) protein located on the cell surface receptors initiate virus entry into host cells, the NA(Neuraminidase) proteins act as an enzyme catalyzing viral replication. The other influenza virus types C and D viruses have only one surface glycoprotein, hemagglutinin–esterase fusion (HEF) The segmented viral genome of influenza can cause reassortment as an exchange of different RNA segments between different viral strains resulting in antigenic shift and cause possibles new pandemic strains. And are prone to high genetic variability due to frequent mutations (Wu & Wilson, 2020)

In Africa, multiple subtypes and lineages often co-circulate within a single season, though the dominant subtype varies annually(Nyamusore et al., 2018).For example, Kenya and South Africa have consistently reported circulation of both A subtypes and alternating B lineages (McMorrow et al., 2015) Rwanda's reports indicate circulation of H1N1pdm09, H3N2, and both B lineages in various seasons, Rwandan surveillance (2008–2010) found that of 377 positive cases, 71.8% were H1N1pdm09, 5.6% H1N1, 7.7% H3N2, and 13.3% influenza B. (Nyatanyi et al., 2012)These findings align with subtype patterns reported in Kenya, South Africa, and other African countries.

2.1.3 Intracellular Replication and Transcription of influenza virus

Influenza virus replication follows a series of well-defined steps within the host cell. The replication of the Virus occurs mainly in the epithelial cells lining the respiratory or intestinal tract. During infection, the virus binds to receptors on the host cell surface and enters the host cell through endocytosis, viral Ribonucleoproteins (vRNPs) are released into the cytoplasm after entry and transported to the nucleus where the viral polymerase transcribes and initiates replication of the viral genome.(Zhu et al., 2023)

Once inside the nucleus, the negative-sense viral RNA (vRNA)is transcribed into positive-sense messenger RNA (mRNA) by the viral RNA-dependent RNA polymerase through a process known as cap-snatching, where short capped primers are derived from host pre-mRNAs(Dou et al., 2018) .In addition to transcription, the polymerase replicates also the genome by synthesizing complementary RNA (cRNA), which acts as a template for generating new vRNA genomes.(Sparrer & Kirchhoff, 2023)

The virus infects generally the upper respiratory tract and trachea as a mild influenza infection whereas the most severe cases and fatal human infections are associated with viral infections in the lower respiratory tract. Influenza virus causes the death of epithelial cells through various mechanisms. (Liang, 2023)Viral replication peaked at ~48 h after infection and declined slowly thereafter, following infection viral shedding occurs about 24-48 before onset of symptoms.(Javanian et al., 2021)

2.1.4 Clinical Manifestation

Typically, incubation period of influenza virus infection is between 18 and 72h, though it may differ from case to case, symptoms usually begin with high fever and chills ,headache, muscle and joint pain, generalized weakness, eye redness and respiratory symptoms like dry cough and sore throat and rhinitis.(Javanian et al., 2021)

While influenza symptoms in children are very similar to those seen in adults, children may experience additional signs such as nausea, stomach pain, otitis media, and vomiting.

2.1.5 Transmission and Pathogenesis

Influenza viruses are primarily transmitted from infectious people to susceptible people through large virus containing respiratory droplets, contact with contaminated surfaces and aerosols produced by talking, coughing, singing, speaking, even breathing normally can also facilitate spread. (Liang, 2023)

The reservoirs of all known subtypes of type A influenza viruses are birds from the orders of Anseriformes and Charadriiformes. The type A virus can also infect mammalian species like humans, dogs, cats. The influenza type B viruses exclusively infect humans, however type c viruses affect swine. Cattle serve as the primary host for type D virus. (Liang, 2023)

The virus infects the upper respiratory tract and can spread to the lower tract in severe cases, causing symptoms such as fever, cough, sore throat, myalgia, and fatigue. In vulnerable populations, such as young children, the elderly, and individuals with comorbidities, influenza can lead to serious complications, including pneumonia, exacerbation of chronic illnesses, and death. (Javanian et al., 2021)

Influenza viral proteins contribute to the lung pathology in humans, with Hemagglutinin (HA) being a key player in the infection process. HA in seasonal influenza A viruses (IAV) binds to a2-6 sialylated glycans which are complexed sugar molecule (glycans) containing sialic acid attached to galactose via alpha 2,6 bond, the complex are present on the epithelial cells of the upper respiratory tract, leading to mild disease that is confined to the upper respiratory system. This mild disease is easily transmitted through nasal discharges containing high viral titers. In contrast, highly pathogenic avian influenza H5N1 (HPAIV) predominantly binds to a2-3 sialylated glycans, allowing it to infect type 2 pneumocytes in the human lungs, often resulting in severe pneumonia.(Caini et al., 2019)

A study using human tracheobronchial epithelial cell cultures found that the influenza A(H1N1)2009 virus with the D222G mutation in its HA protein could infect ciliated bronchial cells. This mutation may change the virus's cell preference and potentially increase pneumonia severity. As a result, it's important to monitor the HA of avian H5N1 viruses for mutations that could affect their pandemic potential, as well as keep track of HA mutations in influenza A(H1N1)2009 that could lead to more pathogenic strains. (Fukuyama & Kawaoka, 2011)

Six influenza pandemics have been experienced from 18th to 20th century: the Asiatic flu (1889-1890), Spanish influenza (H1N1) in 1918/1919, Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968 ,Russian flu between 1977-1979 (H3N8), and H1N1 influenza in 2009.These pandemics involved different strains of influenza virus resulting in varying levels of illness and death(Yıldız, 2020)

Global travel contributes to interregional transmission of influenza viruses, causing the introduction of novel strains into new regions. data from surveillance system confirm that new variants often emerge in East and Southeast Asia and then spread worldwide.(Haider & Hassan, 2025)

2.2 Geographical distribution of influenza

Globally influenza virus circulates, however their display varies across geographical regions and seasons, influenced by population density climate condition, travel and immunity levels of population.(Tamerius et al., 2013)

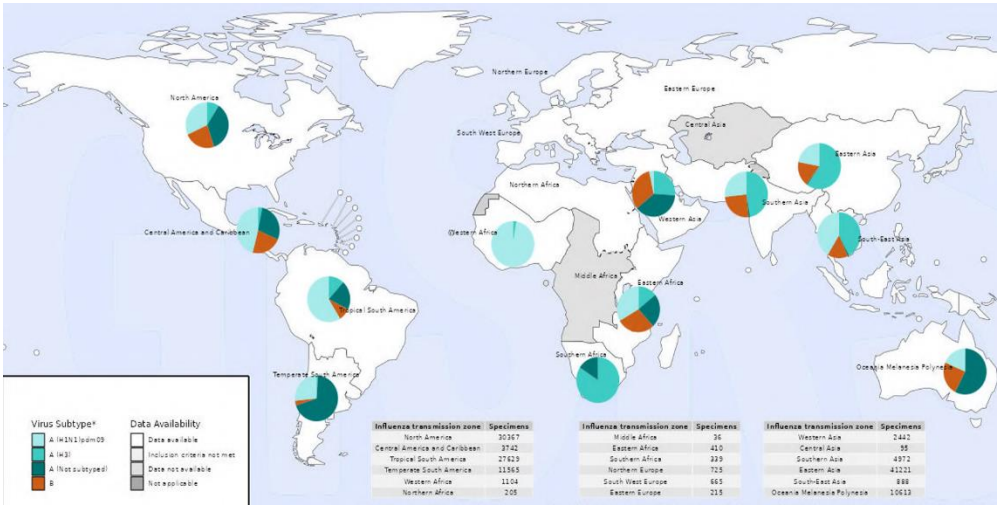
2.2.1Temperate Regions

In temperate climates, in the regions of northern hemispheres, influenza activity is highly seasonal with a peak of transmission during winter seasons (typically November-March) and in the southern hemispheres from May to September. These seasonal peaks align with colder weather, which enhance viral stability in the environment increasing the risk of transmission.(Audi et al., 2020)

Temperate regions of the northern and southern hemispheres are characterized by highly synchronized annual influenza epidemics during their respective winter months .(Tamerius et al., 2013)

2.2.2Tropical and Subtropical Regions

Influenza seasonal characteristics in tropical and subtropical areas are more diverse, some regions experience annual influenza epidemics coinciding with rainy season, or fluctuations of humidity.in sub-Saharan Africa the viral transmission is more variable and less predictable with peaks associated with rainy season, east Africa is often characterized by peaks during the long and short rainy seasons. (Caini et al., 2019)



"Figure 2 Proportions of influenza virus types and subtypes by influenza transmission zones."

(Surveillance, n.d.)

2.2.3Influenza in Africa and Rwanda

While influenza manifests seasonal patterns in temperate regions, its transmission in tropical regions, including much of Africa, shows year-round with peaks often linked to rainy seasons. In Africa, Influenza surveillance reports in the past five years have shown that influenza virus activity seems to vary depending on climate and geographic location, surveillance systems. (WHO, 2017)In Rwanda, seasonal peaks are observed around the rainy seasons, from October -November and February-March and subtype distribution varies by year, though molecular surveillance remains limited(Wane et al., 2012).

2.2.4 Influenza Burden in Africa

In sub-Saharan Africa, Influenza is a recognized contributor to acute respiratory infections. Surveillance studies have shown that influenza contributes significantly to hospitalizations for pneumonia and other respiratory illnesses(McMorrow et al., 2015). The region experiences variable Seasonal patterns linked to humidity and rainfall, and co-infections such as malaria complicate diagnosis and management.(Caini et al., 2019)

2.2.5 Prevalence of Influenza in Africa

Influenza continues to be a significant cause of acute respiratory illness in Africa, contributing to both seasonal epidemics and occasional outbreaks. The overall burden of influenza in Africa is under-estimated due to limited surveillance capacity; however, studies have indicated that influenza is responsible for 10%–20% of influenza-like illness (ILI) and severe acute respiratory infection (SARI) cases presenting at health facilities(McMorrow et al., 2015)

Data from sentinel surveillance sites in sub-Saharan Africa countries such as South Africa, Kenya, Ghana, and Madagascar show consistent annual circulation of influenza A (H1N1pdm09, H3N2) and influenza B viruses, with varying intensity and timing across regions(Owuor et al., 2021) Notably, A study in Ghana found influenza prevalence of 7.4% (Nuvey et al., 2019).In Rwanda, influenza has been detected(Nyatanyi et al., 2012) analyzed data found a 29.6% of prevalence (August 2008–July 2010), Of 377 influenza-positive cases: 71.8 % were A(H1N1) pdm09, 7.7 % A(H3), and 13.3 % influenza B. Cases peaked during the October–November and February–March rainy seasons Findings were pivotal as the first documentation of influenza seasonality in Rwanda.(Nyatanyi et al., 2012)

2.3 Epidemiological and Demographic Patterns Associated with Influenza Subtypes

Influenza impacts all age groups, but surveillance across Africa shows a higher burden of disease among children under five, pregnant women, and those with chronic illnesses, including HIV(Baldo et al., 2016) The epidemiological pattern varies by climate, with equatorial regions showing influenza activity throughout the year and peaks during rainy seasons, and southern regions showing winter seasonality.(Lowen & Steel, 2014)

A study in Ghana by Nuvey *et al*, found that there is a significant evidence indicating a high prevalence(13.3%) of influenza like illness in children under five years of age (Nuvey et al., 2019)

In Rwanda, data suggest influenza cases peak during rainy seasons,(Nyamusore et al., 2018) but demographic data on subtype distribution has yet to be fully analyzed. Further molecular-based studies are needed to clarify these patterns.

2.4 Molecular Surveillance of Influenza and Its Challenges

Molecular based techniques have been essential in identifying circulating influenza virus types and subtypes over the past two decades. The continuous ongoing circulation of highly pathogenic influenza virus show up the need for accurate and rapid diagnosis and subtyping of influenza viruses for surveillance, tracking virus evolution, managing outbreak, and informing public health response.(Wang & Taubenberger, 2010)

The application of Molecular techniques such as reverse transcriptase-PCR, real-time PCR, microarrays and other nucleic acid sequencing-based amplifications, have greatly improved the ability for monitoring, characterization of influenza viruses and surveillance strategies.(Wang & Taubenberger, 2010)

2.4.1 Reverse Transcription Real time PCR

The polymerase chain reaction (PCR) is a basic molecular technique developed in the 1980s by Dr. Kary Mullis. The technique is often referred to as a "molecular photocopier" due to its capacity to identify a specific DNA sequence and quickly and precisely generate a large number of copies.(Jalali et al., 2017)

The reverse transcription polymerase chain reaction (RT-PCR) is known as a highly sensitive method for the quantification of mRNA molecule, the first step in this technique an mRNA

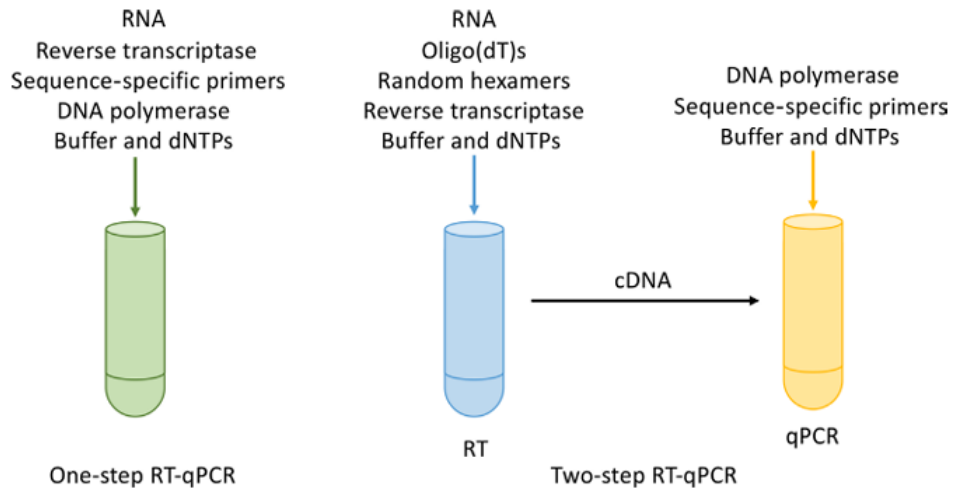
molecule by using reverse transcriptase enzyme is converted to single stranded cDNA, which is converted into a double stranded molecule followed by amplification of DNA. The technique is used for detection of pathogens including viruses and quantification of gene expression levels (Herrington & O'Leary, 1997)

During each PCR cycle, the quantity of amplified DNA is measured in real-time using fluorescent chemistries, which provide a means of quantification. The two most common approaches for generating a fluorescent signal are the use of hydrolysis probes, such as TaqMan® probes, and double-stranded DNA binding dyes like SYBR® Green dye. The choice of fluorescent chemistry depends on various factors, including the specific application, cost considerations, and whether the assay is single plex (one target) or multiplex (multiple targets). (Ponchel et al., 2003)

2.4.1.1 One-Step vs. Two-Step RT-PCR

Reverse transcription PCR can be done in either a one-step or a two-step assay, each offering distinct advantages based on the specific requirements of the study. In the one-step method, both reverse transcription and amplification are conducted in a single tube. This method employs genetically modified reverse transcriptase that can support the higher temperatures required for annealing sequence-specific primers. The assay is particularly suitable for high-throughput applications and diagnostic settings where repeated quantification of the same gene is important. However, since cDNA synthesized in this method cannot be stored for future use, larger quantities of the original RNA may be required to repeat the assay.(Al-Shanti et al., 2009)

In contrast, the two-step RT-PCR technique involve using two separate tubes for reverse transcription and PCR amplification, each containing its own set of buffers and reagents. This separation enables more flexibility, allowing for optimization of both the reverse transcription and PCR steps. The synthesized cDNA in the first step is typically more stable and can be stored for later use or concentrated for subsequent assays. This method is especially useful for applications where multiple genes need to be quantified from the same sample, or where optimizing amplification efficiency for difficult templates is crucial. (Al-Shanti et al., 2009)



(Sinha & Mann, 2020) *"Figure 3One-step vs Two-step RT-PCR".* (Sinha & Mann, 2020)

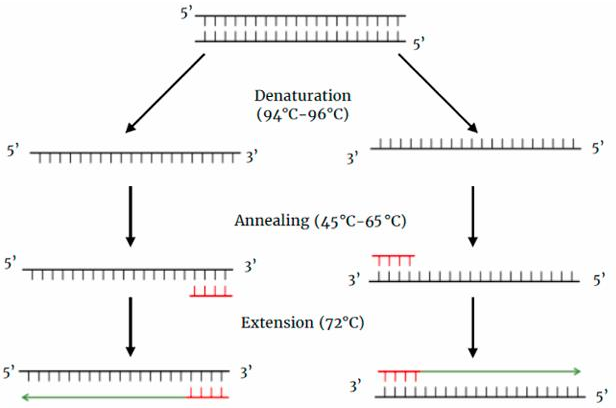
In one-step RT-PCR, cDNA is generated through reverse transcription, followed by PCR amplification of the target sequence, all within a single reaction. In contrast, two-step RT-PCR separates the reverse transcription (RT-PCR) and PCR steps, allowing for the analysis of multiple target sequences in the PCR reaction. (Sinha & Mann, 2020)

2.4.1.2 Real time Reverse transcription principle

PCR is an in vitro technique that mimics a basic form of DNA replication, a natural process that all living cells use to duplicate their genetic material before cell division. It involves multiple cycles of heating and cooling a reaction mixture containing a DNA template, DNA polymerase, primers, and nucleotides. The DNA template is the source of the target sequence. Primers are short nucleotide chains that bind to the specific target DNA through complementary base pairing during cooling, serving as the starting point for DNA polymerase to synthesize a new complementary strand. DNA polymerase is the enzyme responsible for creating new DNA strands that are complementary to the target sequence.(Jalali et al., 2017)

Each cycle of PCR involves three essential steps. First, denaturation occurs, where the reaction mixture is heated to temperatures above 90°C. This high heat breaks the hydrogen bonds between the two strands of the DNA double helix, causing them to separate. Next, during primer annealing, the mixture is cooled to a temperature between 45–65°C, allowing the forward and reverse primers to bind to their complementary sequences on the opposite strands of the DNA. These primers must align with the 3′ ends of the antiparallel strands of the DNA template. Finally, in the extension

step, the temperature is raised to around 72°C, which is the optimal temperature for DNA polymerase activity. The polymerase enzyme then attaches to the primer-template hybrid, using the free nucleotides in the mixture to build a new complementary strand of DNA. After extension, the reaction mixture is heated again to the denaturation step, and the PCR process continues. (Jalali et al., 2017)



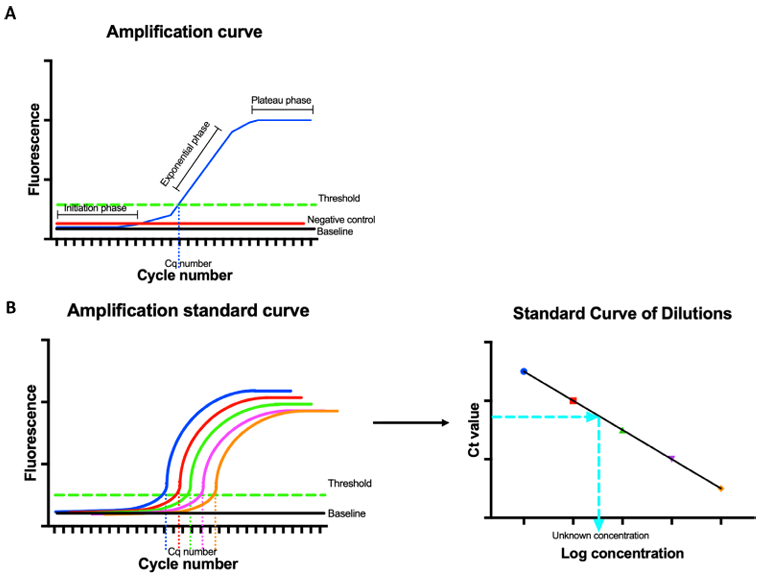
(Jalali et al., 2017)

"Figure 4PCR Steps" (Jalali et al., 2017)

FIGURE 6 First cycle of PCR. In the denaturation step, the DNA is heated to temperatures above 90°C, causing the two strands of the target DNA sequence to separate. The reaction is then cooled to 45–65°C, allowing the primers to bind to their complementary sequences on the template DNA. During the extension step, the temperature is raised to 72°C, enabling the DNA polymerase to synthesize a new strand of DNA that is complementary to the template strand. (Jalali et al., 2017)

2.4.1.2 Real time PCR phases

As the reaction progresses, real time PCR allows for detection and quantification of the targeted DNA, Real-time detection of the PCR cycle produces an amplification curve that includes initiation, exponential, and plateau phases. (Jalali et al., 2017)



(Sinha & Mann, 2020)

"Figure 5Real time PCR phases"

Figure 6(A) The amplification curve is generated in real-time, shows fluorescence detection (blue curve) starting at low levels (baseline). As the reaction progresses into exponential phase, a threshold (green line) is established, the CT value used to quantify is determined when the curve crosses the threshold. Negative controls remain near the baseline. (B) A standard curve is created by plotting Cq values from a serial dilution of known concentrations against their logarithmic values. This allows accurate quantification of unknown target sequences based on their Cq values, as shown by the hashed cyan line.(Sinha & Mann, 2020)

In Africa, real-time reverse transcriptase PCR (RT-PCR) is the primary molecular tool used for influenza surveillance due to its high sensitivity, specificity, and practicality in resource-limited settings(Nabakooza et al., 2022). In Rwanda, national influenza surveillance uses RT-PCR to confirm influenza virus presence and determine subtypes at sentinel sites

RT-PCR is the standard technique used in detection and subtyping influenza viruses, it enable rapid identification of influenza A subtypes (e.g., H1N1pdm09, H3N2) and B lineages (B/Victoria, B/Yamagata) (Rodrigo & Méndez, 2012). This method can be used on clinical samples laboratory diagnosis and with its rapid turnaround time facilitate outbreaks investigations of respiratory illness. PCR assay should also be used in influenza virus type A or B identification on individuals with influenza like illness symptoms by detecting viral RNA in respiratory samples and detect the

subtype of human influenza A viruses potentially novel or newly evolving influenza A virus strains.(World Health Organization, 2011)

However, this method does not provide detailed genetic sequence data or information on minor genetic variations or reassortments. (Wang & Taubenberger, 2010)

Challenges to molecular surveillance in Africa include Limited reagent and equipment availability leading to inconsistent testing capacity. High operational costs for maintaining molecular diagnostics in routine surveillance.(Ashenafi et al., 2024)

By concluding most available data on influenza prevalence are now outdated with previous studies conducted in the past decade, have provided minimal insights into the specific demographic and socio-economic risk factors that may influence susceptibility to influenza infection in Rwanda. Vulnerable groups such as children under five, the elderly, individuals with chronic illnesses remain underrepresented in existing data. By generating updated prevalence data and investigating the distribution of influenza across these high-risk groups, the proposed study aimed to fill critical gaps in knowledge. This will support evidence-based strategies for influenza prevention, targeted interventions, and resource allocation, ultimately strengthening national preparedness and response efforts.

CHAPTER 3 RESEARCH METHODOLOGY

3.1 Study setting

This study was conducted from June 2025 to July 2025 across a network of sentinel surveillance health facilities located throughout Rwanda's five administrative regions: the city of Kigali, Northern, Western, Southern, Eastern provinces. These health facilities are integrated into the national influenza surveillance system coordinated by the Rwanda Biomedical Centre (RBC) and were selected based on their ability to identify cases of influenza-like illness (ILI) as well as their capacity to collect and preserve clinical specimens for molecular testing.

The selected facilities included the university teaching hospital of Kigali (CHUK) and Kibagabaga hospital in the city of Kigali, Gihundwe hospital in the western province, Ruhengeri Referral Hospital in the Northern Province, Kibungo referral Hospital in the Eastern Province, and the University Teaching Hospital of Butare CHUB in the Southern Province, this broad geographic coverage allowed the study to capture data from all Rwandan regions, ensuring national representation.

3.4 Study population

The study population comprised a total of 320 samples collected from patients of all ages presenting symptoms of influenza like illness (ILI) during the study period. ILI is defined as an acute respiratory infection characterized by a measured fever of ≥38°C and cough, runny nose, sore throat, muscle pains, headache, sneezing, and feeling tired**,** with symptom onset within the preceding 10 days. This definition is consistent with World Health Organization (WHO) and national surveillance guidelines.

3.5 Sampling Strategy

Nasopharyngeal samples were obtained from patients who presented with symptoms of influenza-like illness (ILI) at a designated influenza surveillance site. This study employed a case series approach, where data were systematically collected from all patients meeting the inclusion criteria, which required documented ILI symptoms and clinical information. The case series included a consecutive sample of ILI cases until the predetermined sample size was reached. These samples were collected as part of an ongoing surveillance program, where patient data and clinical information, including ILI symptoms, were systematically recorded on a case investigation form.

3.5.1 Sample size

A total of 320 nasopharyngeal swab samples were collected for the study. The minimum required sample size was calculated using the single proportion formula (Scalex SP calculator) assuming an estimated influenza prevalence of 29.6% as reported in prior studies conducted in Rwanda (Nyatanyi et al., 2012) a 95% confidence level (Z = 1.96), and a margin of error of 5%. The calculated minimum sample size was 321; therefore, the planned collection of 320 samples provided sufficient statistical power for robust subgroup analyses and improve precision of estimates (Pak & Oh, 2012)

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

(Naing et al., 2022)

3.6 Inclusion criteria

The study included laboratory samples from both male and female patients of all ages who presented with symptoms of influenza-like illness (ILI). To be eligible for inclusion, the samples had to be collected from patients exhibiting typical ILI symptoms such as fever, cough, sore throat, fatigue, runny nose, and headache. These samples were obtained from patients who visited the designated influenza surveillance site during the study period, where clinical data confirming the presence of ILI symptoms were recorded

3.7 Exclusion criteria

Samples were excluded from the study if they came from patients who did not show symptoms consistent with influenza-like illness.

3.8 Study design

This study was a cross-sectional study with a quantitative research approach aimed at detecting and genotyping circulating influenza virus subtypes among samples collected from patients presenting with influenza-like illness (ILI) in selected health facilities in Rwanda. This design was used to facilitate timely detection and subtyping of the virus, the approach ensured efficient data collection and accurate identification of influenza subtypes. The samples were collected with case

investigation forms to capture essential demographic and clinical data, and geographical distribution of influenza subtypes.

3.9 Data and sample collection

The data collection period lasted for 4 weeks (June 2025 - July 2025). Following the approval from national reference Laboratory of Rwanda, we reviewed the clinical and demographic data of patients whose samples were already collected as part of routine surveillance from January 2025 to March 2025.The data collected included age, sex, location, and symptoms which were recorded on case investigation forms. Nasopharyngeal swab samples had been obtained from each patient at the time of their initial presentation, using a sterile, single-use swab. These specimens were immediately placed into Viral Transport Medium (VTM), labeled with a code number for sample identification, and stored at 4°C before transportation. All samples were transported under cold chain conditions to the National Reference Laboratory (NRL) in Kigali for further analysis.

3.10 Laboratory testing

Viral RNA was extracted in the laboratory using Maxwell® RSC viral TNA (Promega kit) on automated Maxwell®RSC 48 Instrument following the manufacturer's protocol. A Flu SC2 multiplex Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to determine influenza virus types A and B and to identify subtypes of detected influenza type A strains (H1N1pdm09, H3N2) and lineages of influenza B (Victoria and Yamagata). specific primers and probes were used in the amplification process. Positive and negative controls were included on each PCR run to ensure the validity of the results. All laboratory procedures followed standard operating procedures and WHO recommended guidelines for influenza virus detection.

3.10.1 RNA Extraction from Nasopharyngeal Swab Samples Using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit

Extraction of viral ribonucleic acid from nasopharyngeal swab samples was performed using Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega) with an automated Maxwell® RSC 48 Instrument. The kit included a Lysis buffer, proteinase K and nuclease free water. As the used samples were preserved at -80°C, they were firstly thawed at room temperature or on ice, followed by a 10 seconds vortexing procedure to ensure complete mixing prior to extraction.(CDC/NCIRD/Influenza Division, 2024)

Extraction Reagents used were prepared in biosafety cabinet level two, to initiate cell lysis 300 µL of lysis buffer was added into sterile Eppendorf tube which disrupt the cell membrane and nuclear envelope and prevent the degradation of RNA by ribonucleases (RNases) followed by addition of 30 µL of Proteinase K to digest proteins including nucleases that could compromise the integrity of RNA.Subsequently,140 µL of each nasopharyngeal sample were added into the tube, This mixture was vortexed and short spined and incubated at 72°C on a heater for 10 min to allow complete lysis process. During the incubation step, Maxwell® RSC system was prepared, ensuring proper placement of RSC cartridges in the Deck Tray and dispensing 75 µL of nuclease-free water to each elution tube. After the incubation phase, the lysate was carefully transferred into respective the cartridges for automated extraction in the Maxwell® instrument. The purification process, which includes washing and elution, was carried out following the instructions in the kit's technical manual. Following a 30-minute run, the extracted RNA was ready for downstream applications. (CDC/NCIRD/Influenza Division, 2024)

3.10.2 Principles of the procedure

The Flu SC2 Multiplex Assay is based on real-time reverse transcription polymerase chain reaction (rRT-PCR) principle. The Assay use three primer/probe sets combinations (InfA, InfB, and SC2) design for amplification and detection of the RNA sequences target of influenza A virus, influenza B virus, and SARS-CoV-2 virus, respectively. The assay also includes a primer and a probe set to detect the human RNase P gene (RP)as an internal control to confirm specimen quality and nucleic acid extraction. Primers and probes for the detection of influenza A viruses were chosen to bind to a well conserved region of the matrix (M1) gene while those selected for detection of influenza B viruses were chosen from a conserved region of the nonstructural 2 gene (NS2). The assay was designed as multiplex reaction runned in a single well, for detection and differentiation of RNA from SARS-CoV-2 virus, influenza A viruses, and/or influenza B viruses. nucleic acids regions extracted from upper and lower respiratory samples served as template for reverse transcription were complementary to the oligonucleotide primers were reverse transcribed into cDNA and amplified by polymerase chain reaction, using an Applied Biosystems 7500 Fast Dx Real Time PCR Instrument operated with SDS version 1.4.1 software. (CDC/NCIRD/Influenza Division, 2024)

If the nucleic acids target were present in the sample, amplification occured, and the probe(s) annealed to specific complementary sequences located between the corresponding forward and reverse primers during the PCR process. During the extension phase of the PCR, the DNA polymerase enzyme with its the 5' nuclease activity of DNA polymerase cleaved the probe bound to the specific target, causing the separation of reporter dye from the quencher dye, generating a fluorescent signal which indicates the presence of specific viral RNA. Each virus Probes emitted a fluorescent signal at different wavelengths, enabling the instrument to differentiate between the signals. With each cycle, additional reporter dye molecules were cleaved from their respective probes, increasing the fluorescence intensity. The applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4.1 software continuously measure the intensity of fluorescence. Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.(CDC/NCIRD/Influenza Division, 2024)

3.10.3 Master mix Preparation for Primers and Probes

Tube 1: CDC Flu SC2 Multiplex Assay: Forward and Reverse Primers

Name	Description	Oligonucleotide Sequence (5' to 3')	Concentration
InfA-F	InfA For1	CAA GAC CAA TCY TGT CAC CTC TGA C	3.33 µM
	InfA For2	CAA GAC CAA TYC TGT CAC CTY TGA C	3.33 µM
InfA-R	InfA Rev1	GCA TTY TGG ACA AAV CGT CTA CG	5.00 µM
	InfA Rev2	GCA TTT TGG ATA AAG CGT CTA CG	1.67 µM
InfB-F	InfB For	TCC TCA AYT CAC TCT TCG AGC G	6.67 µM
InfB-R	InfB Rev	CGG TGC TCT TGA CCA AAT TGG	6.67 µM
SC2-F	SC2 For	CTG CAG ATT TGG ATG ATT TCT CC	6.67 µM
SC2-R	SC2 Rev	CCT TGT GTG GTC TGC ATG AGT TTA G	6.67 µM
RP-F	RNase P For	AGA TTT GGA CCT GCG AGC G	6.67 µM
RP-R	RNase P Rev	GAG CGG CTG TCT CCA CAA GT	6.67 µM

"Figure 6Primers and probes"(CDC/NCIRD/Influenza Division, 2024)

For the amplification process a master mix was prepared, including the necessary reagents such as specific primers, fluorescent probes, a 2X reaction buffer, Superscript III Reverse Transcriptase, Platinum Taq polymerase, and the 2X reaction mix. CDC CAT Flu SC2PPB-EUA primers and probes were initially provided in a dried state and required rehydration prior to aliquoting and use. The combined primer/probe mixtures for each marker set (e.g., Influenza A, H1, H3) were then

aliquoted and stored at -20°C or below until needed for RT-PCR assays.(CDC/NCIRD/Influenza Division, 2024)

The preparation of Master mix was done in a separate clean room under a level two biosafety cabinet. Within this space, a 1.5 mL sterile, nuclease-free tube was labeled for the Flu SC2 Multiplex Assay reaction master mix. The total number of reactions to be prepared, including samples and controls, was calculated in advance. Following this, the required amount of each reagent was calculated by multiplying the number of reactions by the specified volume for each reagent. This approach ensured that an adequate amount of master mix was prepared to accommodate all samples and controls while maintaining consistent reagent concentrations throughout the reactions. Excess master mix reaction was made to allow for pipetting errors, calculations were made as follows:(CDC/NCIRD/Influenza Division, 2024)

Step #	Reagent	Volume of Reagent added per reaction
1	Nuclease free water	N×7.75 µL
2	FluSC2 combined primer mix	N×3 µL
3	FluSC2 combined probe mix	N×3 µL
4	Taqpath 1-step multiplex master mix	N×6.25 µL
	Total volume	N×20.0 µL

Table 1Master mix preparation(*SOP-Influenza FINAL) (1) (1)*, n.d.)

The required reagents were dispensed into the labeled 1.5 mL tube, and the reaction mixtures were mixed by pipetting up and down five times. Care was taken to avoid vortexing. After mixing, the tube was then briefly centrifuged for 15 seconds at 2000 x g to ensure the contents were collected at the bottom of the tube, and then placed on a cold rack. Following this, the reaction strip tubes or plates were arranged in a 96-well cooler rack. A 20 µL aliquot volume of the prepared master mix was dispensed into each designated sample and control wells. Prior to moving the setup to the nucleic acid handling area, the NTC (no-template control) reactions were prepared for NTC well in the assay preparation area. For this, 5 µL of molecular-grade nuclease-free water was pipetted into NTC sample well, and once all wells were prepared we used adhesive film and PCR lid to seal the entire reaction plate, the sealed plate was taken to the sample loading area in the extraction room.(CDC/NCIRD/Influenza Division, 2024)

3.10.4 Sample addition

Extracts were first removed from the refrigerator and the adhesive seal covering the PCR plate or strips was carefully removed. The Eppendorf tubes containing the extracts were opened, and 5 µL of each extract was carefully dispensed into its designated well. To avoid cross-contamination, pipette tips were changed after every addition.

For quality control 5 µL of the negative control (NC) and positive control was added to its corresponding well, once all samples and controls were loaded, the PCR plate or strip was sealed with a new adhesive film and PCR lid to ensure all wells were securely covered. Finally, the sealed plate was transferred to the post-PCR room for amplification.(CDC/NCIRD/Influenza Division, 2024)

3.10.5 Plate Setup on PCR machine

Before initiating the run, the sealed PCR plate was first short spinned at 2000 rpm for 10 seconds to ensure proper mixing and eliminate air bubbles. The Bio-Rad CFX96 Real-Time PCR Instrument was then turned on. After powering on the connected computer, the Bio-Rad CFX96 software was launched, the appropriate protocol template, named "FLU-SC2 Multiplex Template," was selected, Finally, the cycling conditions were reviewed and set as required for the amplification process.(CDC/NCIRD/Influenza Division, 2024)

3.10.6 RT-PCR Amplification and Detection:

After sealing the PCR plate, the run was programmed on the Bio-Rad CFX96 machine starting with UNG incubation at 25°C for 2 minutes, reverse transcription at 50°C for 15min, and activation at 95°C for 2min , followed by 45 cycles of denaturation at 95°C for 15 sec and annealing/extension at 55°C for 30 sec. Fluorescent dyes (FAM, VIC, ROX, and CY5) were assigned to detect InfA, InfB, SC-2, and RNP targets, respectively. (CDC/NCIRD/Influenza Division, 2024)

Figure 7PCR Steps on a Biorad Instrument

3.10.7 Results Interpretation:

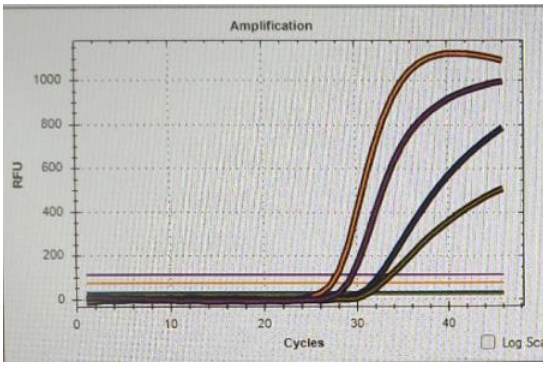
The results of the Influenza and SARS-CoV-2 multiplex PCR test were interpreted by evaluating several controls and sample characteristics. The Positive Control (PC) contained synthetic DNA representing known virus strains and was used to verify the functionality of the PCR reagents and equipment.

For a valid result, the Positive Control containing known synthetic DNA showed positive amplification for all targets (Influenza A, Influenza B, SARS-CoV-2, and RNaseP) within the expected cycle threshold (Ct) range, with Ct values below 33. This confirmed that the test was working as expected.

In addition, the Negative Control (NC) was included to ensure there was no contamination in the reagents or equipment. The Negative Control showed no amplification signals for any of the targets. Similarly, the No Template Control (NTC) also showed no amplification signals, further confirming that there was no contamination from external sources in the test setup.

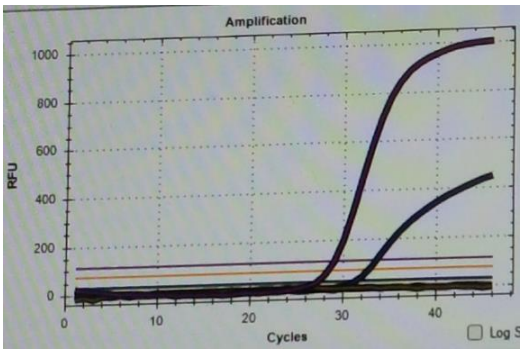
Furthermore, the RNaseP control, which is a human gene used as an internal control, showed a positive amplification trace with a Ct value below 35. This served as an indicator that the sample quality was good and that the nucleic acid extraction was successful.

For the test samples, the interpretation was straightforward. If a sample showed amplification for any of the virus targets (Influenza A, Influenza B, SARS-CoV-2), it was considered positive for the respective virus. All samples had to present a positive amplification trace for RNP below Ct of 35. If no amplification was observed for the virus targets but amplification occurred for RNaseP, the sample was valid and negative for the viruses tested.



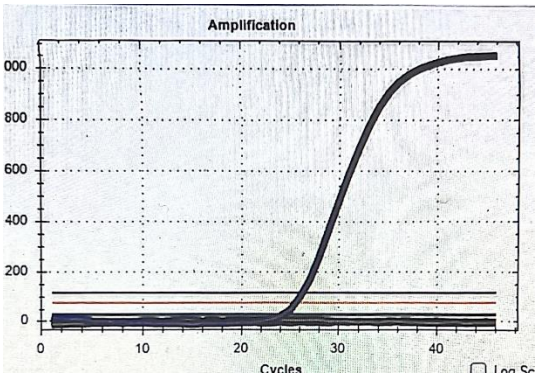
Fluor	Target	Content	Sample	Cq
Cy5	RP	Unkn		29.44
FAM	INF A	Unkn		30.45
ROX	SC2	Unkn		27.19
VIC	INF B	Unkn		30.99

Figure 8Positive Control Results on Biorad



Fluor	Target	Content	Sample	Cq
Cy5	RP	Unkn		29.05
FAM	INF A	Unkn		31.39
ROX	SC2	Unkn		N/A
VIC	INF B	Unkn		N/A

Figure 9Influenza A positive result



Well	Fluor	Target	Content	Sample	Cq
D03	Cy5	RP	Unkn		26.22
D03	FAM	INF A	Unkn		N/A
D03	ROX	SC2	Unkn		N/A
D03	VIC	INF B	Unkn		N/A

Figure 10 Influenza A Negative Result

3.11 Influenza subtyping

Subtyping of Influenza A samples was performed using real-time PCR. Samples positive for Influenza A or B were further tested using extracted RNA to identify subtypes such as H1, H3, H5a, H5b, Swine Flu A, Swine H1, and H7. The specific primer/probe mix was used for each marker, and results were interpreted based on the Ct value threshold of <38.00 for positivity.

Quality control procedures, including the use of Negative Template Controls, Positive Controls, and RNase P testing, were implemented to ensure the reliability of results.

3.11.1 Influenza subtyping PCR set up Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Infl A S1	Infl A S2	Infl A S3	Infl A S4	Infl A S5	Infl A S6	Infl A S7	Infl A S8	Infl A S9	Infl A S10	Infl A NC	Infl A PC
B	AH1 S1	AH1 S2	AH1 S3	AH1 S4	AH1 S5	AH1 S6	AH1 S7	AH1 S8	AH1 S9	AH1 S10	AH1 NC	AH1 PC
C	AH3 S1	AH3 S2	AH S3	AH3 S4	AH3 S5	AH3 S6	AH3 S7	AH3 S8	AH3 S9	AH3 S10	AH3 NC	AH3 PC
D	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a	AH5a

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	NC	PC
E	AH5b S1	AH5b S2	AH5b S3	AH5b S4	AH5b S5	AH5b S6	AH5b S7	AH5b S8	AH5b S9	AH5b S10	AH5b NC	AH5b PC
F	Sw A S1	Sw A S2	Sw A S3	Sw A S4	Sw A S5	Sw A S6	Sw A S7	Sw A S8	Sw A S9	Sw A S10	Sw A NC	Sw A PC
G	Sw AH1 S1	Sw AH1 S2	Sw AH1 S3	Sw AH1 S4	Sw AH1 S5	Sw AH1 S6	Sw AH1 S7	Sw AH1 S8	Sw AH1 S9	Sw AH1 S10	Sw AH1 NC	Sw AH1 PC
H	AH7 S1	AH7 S2	AH7 S3	AH7 S4	AH7S5	AH7 S6	AH7 S7	AH7 S8	AH7S9	AH7 S10	AH7NC	AH7 PC

"Table 2Subtyping PCR setup plate"(*SOP-Influenza FINAL) (1) (1)*, n.d.)

3.11.2 Influenza subtyping results Interpretation

Influenza A or B positive results was indicated by amplification with a CT value < 38.00, while Ct values > 38.00 suggest that the virus was not detected. Positive amplification of H1 gene indicated H1N1 subtype while H3 signals H3N2.Detection of H5 suggests an avian influenza strain whereas H7 indicates H7 subtype presence. The RNase P gene, a human control gene, should always show amplification with a Ct value < 38 to confirm sample quality. If RNase P is not detected, the sample should be re-extracted. Negative Template Controls (NTC) should show no amplification to rule out contamination, while Positive Controls (PC) should confirm the assay's functionality. Inconclusive results, such as a positive RNase P but negative influenza detection, suggest the need for sample re-extraction and retesting. The results interpretation helped for tracking influenza trends, identification of circulating strains types and subtypes.(*SOP-Influenza FINAL) (1) (1)*, n.d.)

3.11.3 Influenza A subtyping Results validation and interpretation

Flu A	H1	H3	H5a and H5b	Sw-FlA and Sw-H1	H7	Results Interpretation	Special guidance
+ <38.00Ct	+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	Flu A/H1N1	N/A
+ <38.00Ct	− ≥38.00Ct	+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	FlA/H3N2	N/A
+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	FluA/H5	H5a and H5b must be positive
+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	+ <38.00Ct	− ≥38.00Ct	FluA/H1N1 Pdmo9	Sw-FluA and Sw-H1 must be positive
+ <38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	− ≥38.00Ct	+ <38.00Ct	FluA/H7	N/A

"Table 3 Influenza A subtyping Resuts validation"(*SOP-Influenza FINAL) (1) (1)*, n.d.)

3.12 Ethical considerations

Ethical approval for this study was obtained from the CMHS institutional review board (IRB) (No. 719/CMHS IRB/2025). A research identification was given to each sample to assure the confidentiality of participants and used solely for research purposes. All procedures were conducted in accordance with the Declaration of Helsinki and Rwandan regulations on research on human subjects.

CHAPTER 4: RESULTS

Analysis of results were done using SPSS Version 25.0 for 64-bit systems

4.1 Prevalence of influenza virus

Samples analyzed in this study were collected between January 2025 and March 2025 through six health facilities-based surveillance system as follow:

Study site	**Number of samples**
Kibungo referral hospital	122
Gihundwe hospital	105
CHUB	26
CHUK	2
Kibagabaga hospital	25
Ruhengeri hospital	40
Total	320

Table 4 prevalence of influenza virus infection

INFLUENZA STATUS	**FREQUENCY**	**PERCENT**
NEGATIVE	288	90
POSITIVE	32	10
TOTAL	320	100

The laboratory results showed that of the total 320 samples tested from patients presenting ILI, only 32 (10.0%) tested positive for influenza virus infection. (Table 1) The majority of the samples (90%) did not show evidence of influenza virus infection. These findings suggest that influenza is relatively less prevalent in the tested population with a small proportion tested positive.

The figure shows varying influenza prevalence across different study sites in Rwanda. CHUK had the highest prevalence at 100%, although this was based on only two samples. Kibungo Hospital

had a higher prevalence at 16.39%, while CHUB had 11.54%. Gihundwe and Kibagabaga Hospitals had lower prevalences of 5.71% and 4.00%, respectively. Ruhengeri Hospital showed no cases, resulting in a prevalence of 0.00%. These findings indicate regional differences in influenza prevalence across the hospitals.

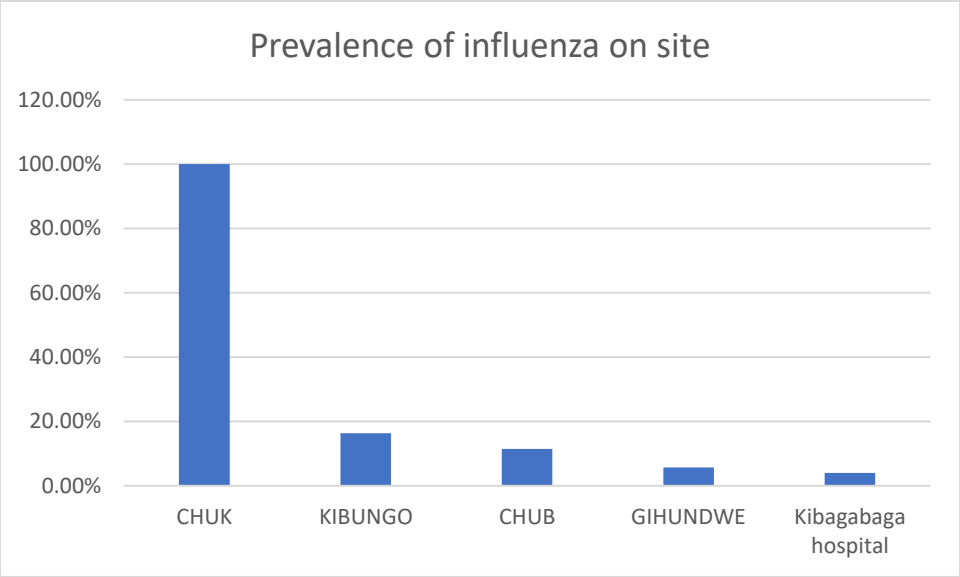


Figure 11represents the prevalence of influenza according to study sites

4.2 Distribution of Influenza virus types and subtypes

The results of this study demonstrate that influenza type A was the only influenza virus type identified in the 320 samples tested. Among the 32 influenza-positive samples, all were classified under the H1N1pdm09 subtype. No other influenza subtypes were detected in the samples analyzed. As such, the predominant influenza virus subtype in this dataset is H1N1pdm09, reflecting its exclusive presence in the positive samples. This finding provides important insight into the distribution of influenza subtypes within the sampled population, emphasizing the dominance of the H1N1pdm09 strain in this study.

Influenza types/subtypes	Number of samples(N)	Percentages (%)
Influenza type A	32	10
H1N1pdm09(subtype of type A)	32	10
Other subtypes	0	0
Negative (no influenza detected)	288	90
Total	320	100

Table 5 Influenza virus types and subtypes

4.3 Epidemiological and demographic patterns associated with detected subtype

4.3.1 Influenza subtype vs Gender

Among 32 positives samples 22(13.3%) were females while 10(6.5%) were males (Table 6) The Chi-square test showed a statistically significant association between gender and H1N1pdm09 positivity p = 0.044); with a relative risk (RR) of 2.03 indicating that females are 2.03 times more likely to test positive for influenza than males.

Table 6 Influenza subtypes Vs Gender

Result subtype	Female(n=166)	Male(n=154)	Total(n=320)
H1N1pdm09	**22(13.3%)**	**10(6.5%)**	**32(10%)**
Negative	**144(86.7%)**	**144(93.5%)**	**288(90%)**
Pearson chi-square			**P. value (0.044)**
Relative Risk			**2.03**

4.3.2 Association Between Age Group and H1N1pdm09 Subtype Detection

To explore the association between age group and the presence of the H1N1pdm09 influenza subtype, a Chi-square test of independence was conducted on 320 participants categorized into nine age groups. The results demonstrate that although most H1N1pdm09 cases occurred in age group 1, this trend is proportional to the group's representation in the sample (70%). The Chi-square test (p = 0.837) did not show a significant association between age group and H1N1pdm09 detection.

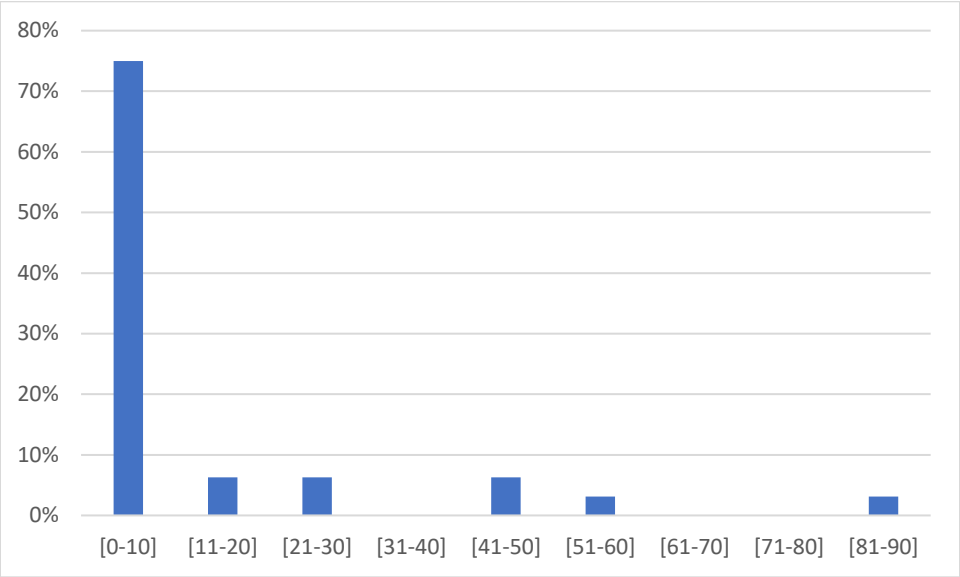


Figure 12 Prevalence of influenza according to age distribution

4.3.3 Association Between study site and H1N1pdm09 Subtype Detection

This table indicates a clear geographic variation in the detection of H1N1pdm09, with the Eastern Province (Kibungo) being the most affected site with 62.5% of all H1N1pdm09 positive cases followed by Gihundwe Hospital (18.8%). Smaller contributions were seen in other hospitals, with CHUB, CHUK, Kibagabaga Hospital, and Ruhengeri Hospital reporting no positive cases. A Pearson Chi-Square test was used to assess whether the distribution of H1N1pdm09 cases differed significantly across the study sites. The Pearson Chi-Square test yielded a value of 31.195 with 5 degrees of freedom and a p-value < 0.001, indicating a statistically significant association between the study site and the likelihood of detecting H1N1pdm09.

Study site	**H1N1pdm09**	**%within H1N1pdm09**	**Negative(n)**	**Total(n)**
Kibungo referral hospital	20	62.5%	102	122
Gihundwe hospital	6	18.8%	99	105
CHUB	3	9.4%	23	26
CHUK	2	6.3%	0	2
Kibagabaga H	1	3.1%	24	25
Ruhengeri hospital	0	0.0%	40	40
Total	32	100%	288	320

Chi-Square Test Results:

Test	P- value
Pearson chi-square	0.000

Table 7 Influenza subtypes Vs Study site

CHAPTER 5: DISCUSSION

5.1 Prevalence of influenza infection

In this study, the overall prevalence of laboratory-confirmed influenza infection was 10.0%, with 32 out of 320 samples testing positive for influenza A. The remaining 90% of samples were negative.

The observed prevalence aligns with some findings reported in similar surveillance studies across sub-Saharan Africa, where influenza positivity among ILI cases generally ranges between 8% and 20%, depending on the region, season, and study population. A study conducted in Kenya reported a positivity rate ranging from 14.6% among ILI cases(Katz et al., 2014) In Rwanda, assuming an estimated influenza prevalence of 29.6% as reported in a study conducted by Nyatanyi *et al*, (Nyatanyi et al., 2012) the observed prevalence is low, suggesting that, as samples were collected from the dry season which may temporarily suppress influenza transmission due to lower relative humidity and higher temperatures, both known to reduce viral survival in aerosols.(Tamerius et al., 2013)

From a public health perspective, a 10% positivity rate can reflect the possibility that other respiratory pathogens, such as rhinoviruses, adenoviruses, or respiratory syncytial virus (RSV), were more dominant during the study period as the samples were collected from patients presenting symptoms of influenza like illness.(Tayachew et al., 2025)

Globally, however, influenza positivity among ILI cases can vary more widely between 25–30% prevalence.(Region et al., 2016)

Interestingly, the low influenza detection rate in this study may also reflect the impact of non-pharmaceutical interventions (NPIs) introduced during the COVID-19 pandemic. Measures such as mask-wearing, and improved hand hygiene have been associated with reduced circulation of influenza viruses in multiple countries.(World Health Organization, 2020) While this study occurred after the most intense phases of COVID-19, behavioral changes in the community may still have affected influenza transmission patterns.

Overall, the 10% prevalence observed is consistent with regional trends in East Africa, though lower than global peaks.

These findings underscore the importance of sustained surveillance to monitor changes in influenza circulation, detect emerging subtypes, and guide vaccine deployment strategies.

5.2 Predominant influenza virus types and subtypes

In this study, Influenza type A with H1N1pdm09 subtype was the only influenza virus type and subtype detected, with 32 samples (10.0%) testing positive for this strain. The remaining 288 samples (90%) were negative for influenza indicating that subtyping was not performed for negative results. The presence of H1N1pdm09 in a small proportion of the samples points to the ongoing circulation of this strain in the study population, with limited viral spread at the time of testing.

The H1N1pdm09 virus is part of the Type A influenza viruses, which are known for their high mutation rates and ability to cause seasonal epidemics and occasional pandemics(Oliveira et al., 2014). The detection of this strain implies current influenza activity in the region and suggests that this virus remains a relevant pathogen for public health monitoring.

Globally, H1N1pdm09 continues to be one of the predominant influenza strains, particularly in seasonal outbreaks. In the United States and other temperate climates, H1N1pdm09 has remained dominant in recent influenza seasons, along with other subtypes like H3N2 (Summary & Surveillance, 2025)Similarly, studies in Africa have also documented the continued circulation of H1N1pdm09 in the post-pandemic era. For instance, a study in Kenya found H1N1pdm09 to be the most prevalent subtype in across multiple seasons, reflecting similar findings to the present study.(Owuor et al., 2021)In South Africa, H1N1pdm09 was found to be the predominant subtype during influenza seasons.(Wolter et al., 2022)

The 10% positivity rate for H1N1pdm09 in this study is within the range of influenza subtypes observed in Africa, However, the absence of other subtypes such as H3N2 or Influenza B which is commonly detected alongside influenza A during seasonal epidemics, may seem unexpected in some contexts, especially in years when multiple strains typically co-circulate. This could be due to regional variation in viral circulation patterns, the timing of data collection as influenza A viruses, particularly H1N1pdm09, often dominate early in the flu season, while Influenza B may peak later.(Nabakooza et al., 2022)

5.3 Epidemiological and demographic patterns associated with detected subtype

5.3.1 Influenza subtype vs Gender

In this study, the association between gender and H1N1pdm09 infection reached statistical significance, as indicated by chi-square Test with a P value of 0.044 and a relative risk of 2.3. The relative risk of 2.03, which we previously observed, now takes on greater importance given the significant p-value from the Chi-Square test. This relative risk suggests a trend toward higher positivity among females. With the Chi-Square test confirming statistical significance, we can now state that females in our study were more than twice as likely to test positive for H1N1pdm09 compared to males.

This finding may reflect underlying biological or behavioral factors. A study by Chweya *et al,* (Chweya et al., 2021)observed a higher frequency of respiratory viral infections in females, which they attributed to factors such as caregiving roles or differences in health-seeking behavior.(Emukule et al., 2023) For instance, women are often primary caregivers and may have increased exposure to infected individuals, particularly children. (Emukule et al., 2023) Additionally, there is evidence suggesting that hormonal differences may influence immune responses to viral infections, potentially making females more susceptible (Statistics Canada, 2022). Although our sample size of 320 was sufficient for the Chi-square analysis, it remains modest for subgroup analyses. We recommend larger, multi-center studies that stratify by both age and sex to clarify these relationships and better inform targeted influenza prevention strategies.

5.3.2 Influenza subtype vs Age

The results of this study revealed that the age group 0–10 years accounted for the highest proportion of H1N1pdm09-positive cases, representing 75% (24 out of 32) of all laboratory-confirmed positives. The age groups 11–20 years and 21–30 years each contributed 6.3% (2 cases) of the total. Notably, no positive cases were observed in participants aged 31–40, 61–70, or 71–80 years. Despite these apparent trends, statistical analysis using chi-square test found no significant association between age group and influenza infection status (p = 0.837), indicating that the observed differences were not statistically meaningful at the 5% significance threshold.

Scientifically, the concentration of H1N1pdm09 infection in younger age groups is epidemiologically plausible and consistent with patterns observed in other influenza studies. Children and adolescents, particularly those under the age of 10, generally have limited prior

exposure to seasonal or pandemic influenza viruses and therefore possess low levels of pre-existing immunity.(C, 2009) Additionally, younger populations are more likely to experience higher viral shedding, have closer contact in school or household settings, and less consistently practice infection control behaviors, all of which contribute to their role in influenza transmission.(Quaggiotto et al., n.d.)

These findings are consistent with previous literature. For instance, McMorrow *et al*, reported that during the 2009 H1N1 pandemic, children under five years of age had the highest influenza-associated hospitalization rates.(McMorrow et al., 2015) Similarly, Baldo *et al*, in Italy found that children under 14 years were disproportionately affected by H1N1pdm09 during the 2010–2011 influenza season.(Baldo et al., 2016) In East Africa, Dawa *et al,* observed higher influenza positivity rates in children under five compared to older populations(Dawa et al., 2018). These studies support the biological plausibility of the current findings and suggest that, while not statistically significant, the pattern observed in this dataset is not random.

In summary, although no statistically significant relationship was identified between age and H1N1pdm09 infection status in this sample, the observed age distribution of positive cases closely mirrors patterns reported in other influenza studies. These findings emphasize the importance of prioritizing influenza surveillance, health education, and vaccination strategies for younger age groups, who play a central role in sustaining transmission during outbreaks.

5.3.2 Influenza subtype vs Study site

The current study found that Kibungo Referral Hospital, located in the Eastern Province, contributed the majority of H1N1pdm09 positive cases (62.5%), followed by Gihundwe Hospital in the Western Province (18.8%). In contrast, the study by Nyatanyi *et al*, analyzed data from the same six sentinel hospitals between 2008 and 2010 in Rwanda, observed no statistically significant difference in influenza-associated SARI hospitalization rates across provinces.(Nyatanyi et al., 2012)

While the current study found that 62.5% of all H1N1pdm09-positive cases were reported from Kibungo Referral Hospital, it is important to note that Kibungo also contributed the largest share of the total sample, with 122 out of 320 participants (38%). Nonetheless, the Chi-square test of independence revealed a statistically significant association between study site and H1N1pdm09 detection ($p < 0.001$), suggesting that the differences in positivity rates were not solely due to

sample size imbalance. The data indicate that Kibungo still had a disproportionately higher number of positive cases relative to its sample share 20 positive cases out of 122 tested (16.4%) compared to only 6 cases out of 105 (5.7%) at Gihundwe, the next most sampled site. The discrepancy could stem from differences in time period, or localized outbreak dynamics. It is also possible that Kibungo experienced a concentrated transmission event during the surveillance window of this study, emphasizing the importance of site-specific and time-sensitive influenza monitoring.

This finding highlights the need for future influenza surveillance studies to carefully account for sampling proportions and population denominators when interpreting site-level differences in positivity rates. The findings underscore also the importance of including multiple sentinel sites in surveillance systems to capture regional heterogeneity in influenza virus circulation. These insights can inform targeted public health responses and resource allocation during influenza seasons. The absence of positive cases in Ruhengeri Hospital is an interesting and unexpected result. This could be due to multiple factors, including effective preventive measures such as early isolation, community awareness.

5.3.3 Study limitations

The study was conducted within a limited time frame, which may not fully capture the seasonal or year-to-year variation in influenza strains. Influenza virus strains can evolve over time, and conducting a longitudinal study would provide more comprehensive insights into these variations across different seasons. Although RT-PCR is a reliable method for detecting influenza subtypes, it may not identify all viral variants, especially those with mutations that alter primer or probe binding sites. To achieve a more comprehensive understanding of the virus strains, further sequencing or alternative detection methods would be necessary. Lastly participants that may have experienced influenza like illness but did not seek medical care were not likely captured in this study.

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study aimed to identify the circulating strains of influenza virus in Rwanda, with a focus on the detection and analysis of influenza types and subtypes using RT-PCR and analysis of associated demographic patterns. Out of 320 tested samples, 10.0 % were positive for influenza type A, and confirmed as H1N1pdm09 subtype. This indicates a seasonal circulation of the H1N1pdm09 subtype during the surveillance period, with no detection of other subtypes of the virus strains such as H3N2 or influenza B.

The data also revealed important epidemiological insights. H1N1pdm09 positivity was significantly higher among females compared to males, suggesting possible biological or behavioral differences in susceptibility or exposure. Although most positive cases occurred in the youngest age group (0–10 years), age was not significantly associated with infection when adjusted for group size. Although no statistically significant relationship was identified between age and H1N1pdm09 infection status in this sample, the observed age distribution of positive cases closely mirrors patterns reported in other influenza studies. These findings emphasize the importance of prioritizing influenza surveillance, health education, and vaccination strategies for younger age groups, who play a central role in sustaining transmission during outbreaks

A marked geographical disparity was observed, with over 60% of cases detected at Kibungo Referral Hospital, pointing to potential localized outbreaks or regional factors influencing transmission.

These findings support the continued need for molecular surveillance of influenza in Rwanda, particularly through targeted sampling, sex-disaggregated analysis, and regionally representative sentinel sites. They also reinforce the importance of integrating laboratory-confirmed subtype data into national influenza surveillance to guide vaccine strain selection, outbreak preparedness, and public health interventions.

6.2 RECOMMENDATIONS

To national influenza surveillance program

The national influenza surveillance system should maintain and expand molecular subtype testing at sentinel sites, especially during peak transmission periods. This includes strengthening laboratory capacity for real-time RT-PCR and sequencing of positive samples to monitor emerging strains. The findings underscore also the importance of including multiple sentinel sites in surveillance systems to capture regional heterogeneity in influenza virus circulation.

To Ministry of Health

Although age was not significantly associated with subtype detection, the high absolute number of cases in children underscores the importance of sustained influenza surveillance in pediatric populations. Health facilities and schools should be prioritized for early detection and prevention efforts.

To Future researcher

Further studies should explore genetic sequencing of H1N1pdm09 isolates to assess mutations, antigenic drift, and potential vaccine mismatch and better understand influenza seasonality in Rwanda.

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Contains both the primer/probe set and positive controls, 500 reactions Catalog # Flu SC2PP-EUA or Primer/probe set, 500 reactions Flu SC2. *2*.

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APPENDICES

UNIVERSITY of RWANDA

COLLEGE OF MEDICINE AND HEALTH SCIENCES

DIRECTORATE OF RESEARCH & INNOVATION

CMHS INSTITUTIONAL REVIEW BOARD (IRB)

Kigali, 27th /August /2025

INGABIRE IYAMUREMYE Liliane
MSc student, CST, UR

Approval Notice: No 719/CMHS IRB/2025

Your Project Title ***"Molecular Genotyping of Influenza Virus strains in Rwanda", which I plan to conduct as part of my master's thesis."*** has been evaluated by CMHS Institutional Review Board.

		Involved in the decision		
			No (Reason)	
Name of Members	**Institute**	**Yes**	**Absent**	**Withdrawn from the proceeding**
Assoc. Prof. Stefan JANSEN	UR-CMHS	X		
Prof. Donatilla MUKAMANA	UR-CMHS	X		
Dr Danilo Melanes ZAMBRANO	UR-CMHS	X		
Assoc. Prof. Peace UWAMBAYE	UR-CMHS	X		
Dr Nuhu ASSUMAN	UR-CMHS	X		
Dr Moussa HAKIZIMANA	UR-CMHS	X		
Dr. Oliva BAZIRETE	UR-CMHS	X		
Dr. Judith MUKAMULIGO	UR-CMHS	X		
Assoc. Prof. Erigene RUTAYISIRE	UR-CMHS	X		
Dr Innocent HAHIRWA	UR-CMHS	X		
Prof. Eugene RUTEMBESA	UR-CMHS	X		
Assoc. Prof Isiaka ABDULLATEEF	UR-CMHS	X		
Prof. Aimable MUSAFIRI	UR-CMHS	X		
Mr Sunday Francois Xavier	UR-CMHS	X		
Assoc. Prof Murererehe Julienne	UR-CMHS	X		

After reviewing your protocol during the IRB meeting of where quorum was met and revisions made on the advice of the CMHS IRB submitted on 22nd August 2025, **Approval has been granted to your study.**

Please note that approval of the protocol and consent form is valid for **12 months**.

REPUBLIC OF RWANDA

Kibagabaga 11th. /08/2025
No2831/HOP. KIBAG/2025

MINISTRY OF HEALTH
KIBAGABAGA LEVEL2 TEACHING HOSPITAL
Po. Box: 6062, KIGALI
Email: kibagabaga.hospital@moh.gov.rw

To: Ms. Liliane INGABIRE IYAMUREMYE
Reg. No: 223027848
COLLEGE OF SCIENCE AND TECHNOLOGY

RE: Your application to conduct research

Dear applicant,

Reference is made to your letter requesting permission to conduct a Research entitled ***"MOLECULAR GENOTYPING OF CIRCULATING INFLUENZA VIRUS STRAINS IN RWANDA."***

From the research Ethic committee, we are pleased to inform you that you are accepted to perform your research within Kibagabaga L2T Hospital. Please note that your study findings or report must be shared/presented to the research committee of the hospital before its dissemination.

Sincerely,

Dr. Ernest MUNYEMANA

Lt. Col

Director General of Kibagabaga Level Two Teaching Hospital

KIBAGABAGA LEVEL II TEACHING HOSPITAL

REPUBLIC OF RWANDA

Kibungo, on 12/08/2025

MINISTRY OF HEALTH
EASTERN PROVINCE
NGOMA DISTRICT
KIBUNGO L2T HOSPITAL
E-mail: info@krh.gov.rw

KIBUNGO LEVEL TWO TEACHING HOSPITAL INSITUTIONAL RESEARCH ETHICS COMMITTEE (KL2TH_IREC)

Name of the applicant(s):

INGABIRE IYAMUREMYE Liliane

Address: Univeristy of Rwanda/College of Sciences and Technology

Approval Notice: N° 060 /KL2TH-IREC/08/2025

Your project entitled "**Molecular Genotyping of Circulating Influenza strains in Rwanda**" has been evaluated by Kibungo L2T Hospital Institutional Research Ethics committee members.

REPUBLIC OF RWANDA MINISTRY OF HEALTH	RUHENGERI LEVEL TWO TEACHING HOSPITAL Po.Box: 57, MUSANZE Website : rrh.gov.rw ruhengeli.hospital@moh.gov.rw	Client centered Service Integrity Teamwork Innovation

13 AUG 2025

Musanze,

Ref. 955 **/ RL2TH /DG/2025**

Liliane INGABIRE IYAMUREMYE

Re: Your request for data Collection

Dear **INGABIRE**;

Reference is made to your letter dated on 06th August , 2025 applying permission for data collection of the research project entitled **"*Molecular genotyping of circulating influenza Virus strains in Rwanda*".**

We have the pleasure to inform you that you are allowed to conduct the above mentioned research project .However you're obliged to have all the required equipments for use and the final project report will be shared with Ruhengeri Level II Teaching Hospital.

Best regards.

Dr MUHIRE Philbert
Director General of Ruhengeri Level Two Teaching Hospital

Cc:

-Chair of ethic committee
-Head of Research and Education department

REPUBLIC OF RWANDA **Gihundwe on 13rd August 2025**

WESTERN PROVINCE **N°20/...../HGDISTRUSIZI/25**

RUSIZI DISTRICT

GIHUNDWE HOSPITAL

E-mail: gihundwe.hospital@moh.gov.rw

To: -Liliane INGABIRE IYAMUREMYE/0788588471

Re: Acceptance Letter to conduct research.

Dear Liliane,

I am pleased to inform you that after reviewing your Approval Letter and Authorization to conduct research on '**Molecular genotyping of circulating influenza virus strains in Rwanda**'. We are excited to have you join our team and are confident that your skills and experience will be an asset to our organization.

For any further clarification, feel free to reach out to me directly at 0786449634 or via email at: edithmukayiranga@gmail.com.

Once again, congratulations, and we look forward to welcoming you to GIHUNDWE District Hospital.

Sincerely,

Dr INGABIRE NADINE
Medical Doctor
RMDC 4694
GIHUNDWE HOSPITAL

Edithe MUKAYIRANGA
Director General of Gihundwe District Hospital.