INFLUENZA VIRUS INFECTIONS AMONG OUTPATIENTS SEEKING HEALTH CARE AT SELECTED HOSPITALS IN MOROGORO, MBEYA AND ZANZIBAR, TANZANIA

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ABSTRACT

Respiratory infections cause significant morbidity and mortality worldwide and are the second leading causes of death in Tanzania. Several studies involving national sentinel surveillance for influenza and epidemiology of influenza have been conducted in Tanzania. However, there is dearth in the information on the aetiology of respiratory diseases. This study screened for influenza viruses in subject with influenza-like illnesses in selected areas of Tanzania. A total of 735 subjects were recruited at hospitals in Morogoro, Mbeya and Zanzibar and the collected sample were screened for influenza viruses using reverse transcription polymerase chain reaction (RT-PCR). Out of the tested samples, 13% (93/735) were positive for influenza virus. Influenza A accounted for 56% (52/93) of the detected viruses. Majority of positive cases (19/93; 20%) were from individuals <9 years and 20 to 35 years old categories. There was a significant difference in the proportion of influenza positive sample by month ($\chi2=67.9$ $p<0.05$). Two peaks of infection were observed from March to May and from November to January. Four representative influenza isolates were sequenced and genetically characterized. The BLAST search showed that A/Tanzania/BMH1674/2019 isolate from Zanzibar had 99.86% nucleotide identity with Texas/109/2019 strain from Texas in United States. All of the three influenza B viruses from Zanzibar and Morogoro were 100% identical and belong to the influenza B victoria lineage. This study have revealed that influenza A virus was the most prevalent in 2019 and most of the isolates in our study are not independent evolution variants, as they shared high nucleotide similarity with characterized reference strains from the neighbouring countries and other part of the world.

DECLARATION

I, FLORA ALFRED MJEMA, do hereby declare to the senate of the Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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Prof. Gerald Misinzo Date

(Supervisor)

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Date

(Supervisor)

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DEDICATION

This work is dedicated to my lovely children Clara, Francis and Clever and to my husband David Beda for their patience, support and hard times they went through during my studies. I also dedicate this work to my parents Alfred Mjema and Anna Msuya for their support and encouragement that brought me up to who I am today. I will always honour you.

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ABBREVIATION AND SYMBOLS

Abbreviation	Descriptive meaning
%	percentage
<	less than
>	greater than
°C	degree Celsius or degree centigrade
µg/mL	microgram per litre
µL	microliter
µM	micromole
AIDS	acquired immunodeficiency syndrome
ALRI	acute lower respiratory tract illness
ARI	acute respiratory infection
CDC	Centre for Disease Control
cm	centimetre
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
H IV	human immunodeficiency virus
HA	haemagglutinin
HEF	hemagglutinin-esterase-fusion
hr	hour
IgG	immunoglobulin G
ILI	influenza-like illness
kg	kilogram
LRTI	lower respiratory tract infection
MDCK	Madin-Darby canine kidney
mg	milligram
mg/l	milligram per litre
mg/L	milligram per litre
mRNA	messenger ribonucleic acid
n	number of samples
NA	neuraminidase
NEP	nuclear export protein
NP	nucleoprotein
PA	polymerase acidic subunit
PB1	polymerase basic one
PB2	polymerase basic two
PCR	polymerase chain reaction
pH	hydrogen ion concentration
RNA	ribonucleic acid
RNPs	ribonucleoproteins
rpm	revolution per minute
RT-PCR	reverse polymerase chain reaction

s	seconds
SACIDS	SACIDS Foundation for One Health
SUA	Sokoine University of Agriculture
URT	United Republic of Tanzania
URTI	upper respiratory tract infection
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Influenza is an infectious respiratory disease of the upper or lower respiratory tract caused by influenza viruses. The majority of respiratory infections are of viral origin and causes high morbidity and mortality worldwide (Tsai *et al.,* 2000; Tregoning and Schwarze, 2010). Frederick (2006) reported that among the respiratory viruses, influenza represents the greatest public health threat in terms of both seasonal and pandemic potential. Influenza viruses induce a severe illness in very young, older individuals and individuals with chronic medical conditions (Troeger *et al.,* 2018). The severity of influenza infection is due to different viral strain circulating in the population. At global level, annual epidemics are estimated to be about 3 to 5 million cases of severe illness and about 290 000 to 650 000 respiratory deaths (WHO, 2018). This makes lower respiratory infections the sixth leading cause of mortality for all ages and the leading cause of death among children younger than 5 years (Troeger *et al.,* 2018). In sub-Saharan Africa, incidence of influenza virus cases varies from 5.1%–25.9% with mortality rate range from 0.1% – 5.3% in different countries (McMorrow *et al.,* 2015). Previous studies in Tanzania reported 8.0% prevalence of influenza viruses (Mmbaga *et al.,* 2012). Respiratory tract infections are the second leading causes of death in Tanzania (Mboera *et al.,* 2018) and account for 12.9% of the total hospital deaths (Kishamawe *et al.,* 2018). In a study among tuberculosis patients, an overall prevalence of respiratory viral pathogens was found to be 20.4% (Mhimbira *et al.,* 2018).

Epidemiological data on respiratory tract infection show seasonal differences according to countries and also vary according to the methods used to detect viral pathogens (Çiçek *et al.,* 2015). Influenza viruses have the following genera; Alphainfluenzavirus (influenza A virus), Betainfluenzavirus (influenza B virus), Deltainfluenzavirus (influenza D virus), Gammainfluenzavirus (influenza C virus), Thogotovirus, Isavirus and Quaranjavirus. Influenza A virus are further classified into subtypes based on the antigenicity of their haemaglutinin (HA) and neuraminidase (NA) proteins on the surface of the virion, while influenza virus B is categorised into two lineages Yamagata and Victoria lineage (WHO, 2018). Most of the pandemic strain of influenza that follow the emergence of a novel virus result from genetic shift that involve new combination of the HA or NA gene between different influenza A subtypes viruses. Influenza respiratory disease is characterized by fever, cough, sore throat, nasal congestion and systemic symptoms such as headache, myalgia, and malaise (James, 2008). On the other hand, the upper viral respiratory tract infections may lead to lethargy and poor feeding (Tregoning and Schwarze, 2010).

Influenza like other respiratory viruses such as COVID-19 spread via contact or aerosol transmission routes. Contact transmission can be through direct (contaminated hands) or indirect (fomites) virus transfer from infected person to susceptible individual (Krutter *et al.,* 2018). Factors influencing spread of influenza virus include environmental factors such as humidity, temperature, crowding of people, and host factors such as receptor distribution throughout the respiratory tract (Krutter *et al.,* 2018). The principal ways in which influenza virus and other respiratory viruses are diagnosed include virus culture technique, immunofluorescence or antigen detection, reverse transcription polymerase chain reaction (RT-PCR) technique and film array respiratory panel (RP) assay. Many people recover with taking in plenty of fluids and resting. Antiviral medications using

amantadine, rimantadine, oselfamivir and zanamivir are used in severe or prolonged viral respiratory infections and most of them are forbidden to infants (CDC, 2017). Currently treatment for severe viral respiratory infection in infants relies on supportive measures such as supplementation of oxygen, monitoring of apnoea, nasogastric tube feeding or intravenous fluids, and mechanical ventilation (Tregoning and Schwarze, 2010).

1.2 Problem Statement and Justification of the Study

Estimates indicate that, globally up to 1.9 million children die each year from acute respiratory infections (ARI) with about 70% of these deaths occurring in Africa and South East Asia (Simoes *et al.,* 2006). Respiratory diseases account for 12.9% of all deaths occurring in hospitals of Tanzania (Kishamawe *et al.*, 2019). Many studies on respiratory diseases have focused on prevalence of viral infections in under 5 years children and in immunocompromised patients (Mhimbira *et al.,* 2018; Rotrosen *et al.,* 2017; McMorrow *et al.,* 2015). Several studies have reported that viruses are the most important causes of upper and lower respiratory tract diseases in infants and young children (Downham *et al.,* 1975; Van woensel *et al.*, 2003; Van gageldonk-lafeber *et al.*, 2007; Symekher *et al.,* 2009). Viruses that are associated with respiratory infections include influenza virus, adenovirus, parainfluenza virus, respiratory syncytial virus, human enterovirus, human coronavirus, human Boca virus and other unidentified viruses (Kurskaya *et al.*, 2018).

Influenza viruses have been long established to be the causes of ARI in the high-income countries, but there is little information in low- and middle-income countries. In Kenya, a prevalence of 11% of influenza like illness (ILI) and 12% of acute lower respiratory tract illness (ALRI) due to influenza virus infections has been reported (McMorrow *et al.,*

2015). In a study involving sentinel surveillance in Tanzania by Mmbaga and colleagues (2012) found that influenza virus was a common contributor to respiratory illness affecting all age groups. Despite the reported cases of respiratory infections (McMorrow *et al.,* 2015; Mmbaga *et al.,* 2012) the information on the role of influenza virus in the aetiology of ARI in low and middle-income countries is lacking. Therefore, it is important to determine the aetiology of influenza viral infections to design appropriate management and control strategies.

1.3 Objectives of the Study

1.3.1 General objective

To determine prevalence of influenza viruses among outpatients seeking health care from hospitals in Zanzibar, Mbeya and Morogoro regions in Tanzania.

1.3.2 Specific objectives

The specific objectives of this study are:

i. To determine the prevalence of influenza viruses in outpatient individuals seeking healthcare from selected health care facilities; and

ii. To determine the diversity and genetic characteristics of influenza viruses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition of Influenza

Influenza is a common acute respiratory infection of humans and a range of animals including birds caused by their exposure to species of influenza virus. Respiratory infection causes high morbidity and mortality worldwide and is the second cause of death in Tanzania (Mboera *et al.*, 2018). Influenza incidence and severity differ from year to year and from season to season (Rotrosen *et al.*, 2017). The most pandemic influenza infection is caused by influenza virus A subtypes.

2.2 Aetiology

2.2.1 Classification

Influenza virus belongs to the family *Orthomyxoviridae* which has the following genera: Alphainfluenzavirus (influenza A virus), Betainfluenzavirus (influenza B virus), Deltainfluenzavirus (influenza D virus), Gammainfluenzavirus (influenza C virus), Thogotovirus, Isavirus and Quaranjavirus (Hutchinson and Yamauchi, 2018). Influenza virus A are further classified into subtypes based on the antigenicity of their HA and NA proteins on the surface of the virion while Influenza virus B is broken down into two lineages namely, Yamagata and Victoria (WHO, 2018). Currently there are 18 known HA subtypes and 11 known NA subtypes (CDC, 2017).

2.2.2 Virus structure

The influenza virion is roughly enveloped spherical in shape. The outer layer of the particle is a lipid membrane which is embedded with glycoproteins known as HA and NA and a small number of ion channel protein (M2). Influenza C and D viruses have only one major surface glycoprotein, the hemagglutinin-esterase-fusion (HEF) protein which corresponds functionally to the HA and NA of influenza A and B viruses (Hutchinson and Yamauchi, 2018). The internal virion core is enclosed by a matrix of M1 protein where inside there are nuclear export protein (NEP) or non-structural protein 2 (NS2) and the ribonucleoprotein (RNP) complex. The ribonucleoprotein complex consists of the viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA dependent RNA polymerase, composed of two polymerase basic and one polymerase acidic subunits (PB1, PB2 and PA) (Nicole and Peter, 2008) (Figure 1).

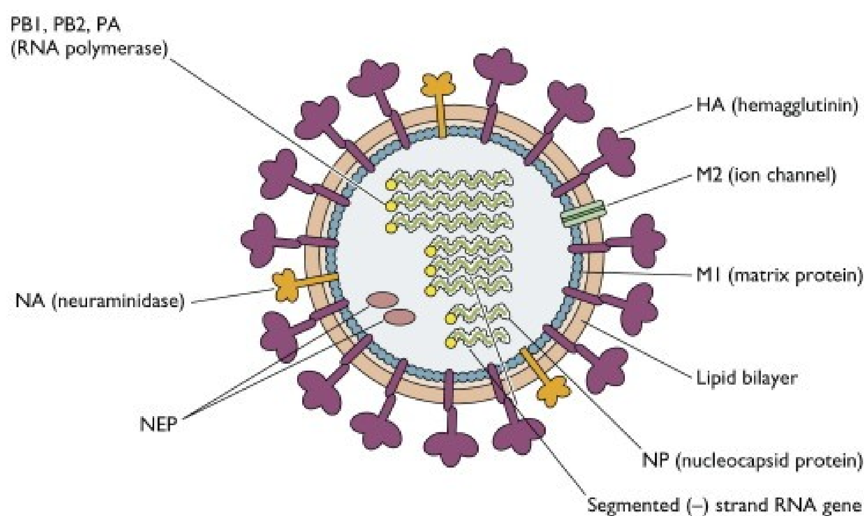

Figure 1: Structure of influenza virus

Source: (https://images.app.goo.gl/3FnYCpy9objYe2cL9)

2.2.3 Genome organisation and replication of influenza virus

The genome of influenza viruses of the Orthomyxoviridae family are single stranded, negative-sense (packaged viral genome is complementary to the mRNA) and segmented RNA.

The viral RNA genome of influenza A and B each comprise of eight segments while influenza C and D viruses have a seven-segment genome (Hutchinson and Yamauchi, 2018). Viral RNA genome segment 4 and 6 codes for the viral HA and NA protein while segment 1, 2 and 3 codes for RNA dependent RNA polymerase protein subunits. Nucleoprotein is coded in segment 5 and other non-structural viral proteins are coded in viral segment 7 and 8 (Nicole and Peter, 2008) (Figure 2).

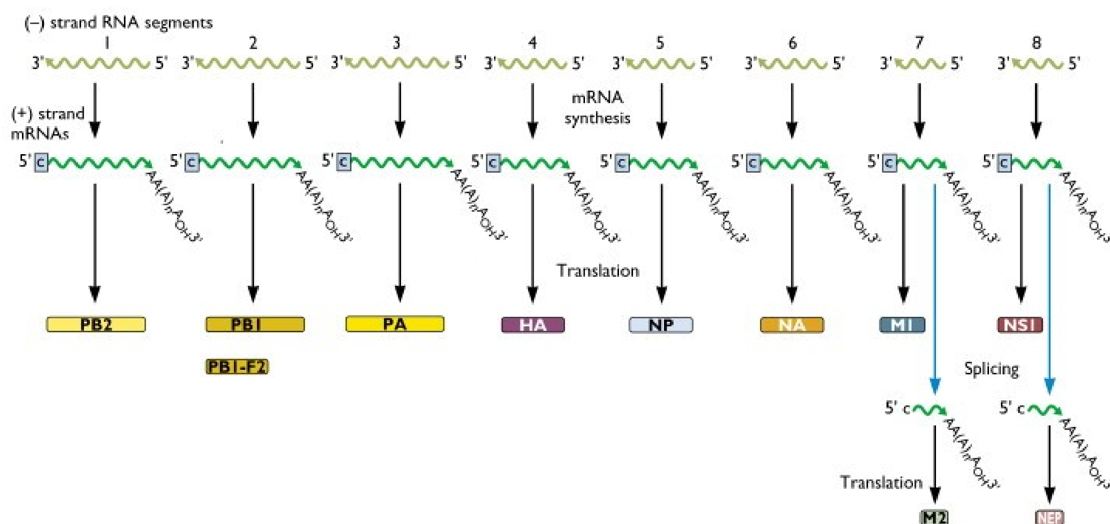

Figure 2: Influenza virus RNA genome

Source: (Google https://images.app.goo.gl/ssT6Ku3g9dAXPEjH7)

Following infection, influenza virion inside the human body diffuses through the mucus layer which protects epithelial cells and binds to the receptors N-acetyl neuraminic acid

present at the terminal position of glycans on glycoproteins and glycolipids at the apical cell surface (Wenjie and Yizhi, 2013). Binding of virion triggers receptor-mediated endocytosis of the virion allowing it to enter inside the cell of the host. Thereafter, the endosomes mature and triggers an influx of hydrogen and potassium ions through the viral ion channel M2.

High level of hydrogen and potassium ions inside the virion causes membrane fusion, matrix disassembly and weakens the interactions between the viral matrix protein M1 and the ribonucleoproteins (RNPs) which encapsulate the viral genome resulting in release of the viral genome into the cytoplasm. Nuclear import factors found in the cytoplasm transport released viral genome to the nucleus (Hutchinson and Yamauchi, 2018).

Inside the nuclear virus uses short capped sequence of a host mRNA to prime transcription of a segment of the viral genome. Viral mRNAs are then translated by host ribosomes and polymerase to nucleoproteins. The nucleoprotein synthesized are then imported back into the nucleus to encapsidate full-length uncapped transcripts of the viral genome into new RNPs (Wenjie and Yizhi, 2013). Newly synthesized RNPs interact with M1 and with the viral nuclear export protein (NEP) which allows them to be exported from the nucleus to the apical plasma membrane of the cell. The trans-Golgi network transports viral transmembrane proteins to the cell surface where the transmembrane proteins, RNPs, M1 and NEP assemble into new virions (Hutchinson and Yamauchi, 2018; Wenjie and Yizhi, 2013) (Figure 3).

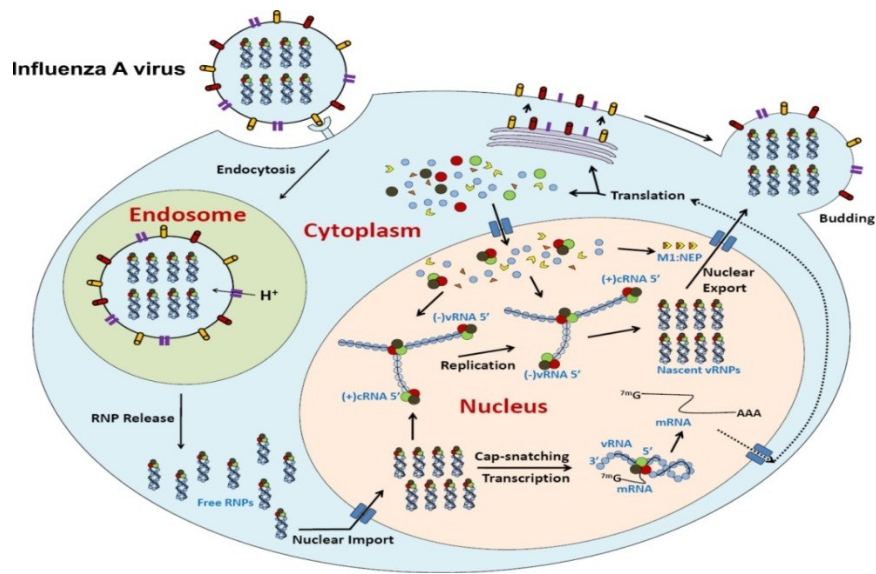

Figure 3: The influenza A virus life cycle. The RNPs are represented by helical hairpins, with the polymerase subunits (red, brown and green) and NP (cyan) shown in different colours. In the nucleus, the viral transcription and replication processes. Source: (Wenjie and Yizhi, 2013).

2.2.4 Antigenic shift and antigenic drift

Due to the segmented nature of the influenza genome, coinfection of a cell with two or more influenza viruses can create reassortant progeny carrying a mixture of genome segments from more than one parental virus (Hutchinson and Yamauchi, 2018). Antigenic shift due to reassortment, recombination or antigenic drift due to error-prone RNA dependent RNA polymerases (mutations) enable these viruses to escape from adaptive immune responses (Wenjie and Yizhi, 2013). However, reassortment of gene segments between genera does not appear to be possible (Nicole and Peter, 2008; Wenjie and Yizhi, 2013).

2.2.5 Viral pathogenesis

The pathogenicity of influenza virus depends on the function of viral proteins and on host immune responses to infection.

Influenza pathogenesis has two phases; the first phase starts immediately post infection with the virus. It is during this phase that much of the clinical signs of the disease is detected including common cold, coryza, cough, hoarseness and rhinitis in upper respiratory tract. For the lower respiratory tract infection, the symptoms include tachypnoea, wheeze, severe cough, breathlessness and respiratory distress (CDC, 2017). The first phase lasts for 3 days' post infection. The second phase starts when the host immune response becomes dysregulated resulting in failure of virus replication control in the first phase which resulting in more inflammation, such as severe pneumonia in humans and sometimes fatal disease in young and older individuals (Anshu and Adrianus, 2019; Satoshi and Yoshihiro, 2011).

2.2.6 Viral survival

The environment factor is a major driver in the survival, evolution and transmissibility of influenza viruses. Survivability of both low-pathogenic and highly pathogenic influenza virus is influenced by physicochemical factors, such as pH, salinity and temperature (Brown *et al.,* 2009). A study in the United States showed that influenza virus persisted for a longer period at low temperature (4–17 $^{\circ}$C), in slightly basic conditions (pH ranging from 7.4 to 8.2) and in fresh to brackish water (salinity ranging from 0 to 20 000 ppm) (Brown *et al.,* 2009).

2.3 Epidemiology of InfluenzaVvirus

2.3.1 Molecular distribution

Molecular epidemiology of influenza viruses become more complex because of its ability to frequently mutate. Studies have reported that influenza A and influenza B viruses are

the most frequently found circulating in human population (Shin *et al.,* 2004 and Van Baalen *et al.,* 2014). In a study in Uganda subtypes H3N2 and H1N1 of influenza A viruses were found to circulate in the human population (Byarugaba *et al.,* 2011). In Tanzania the two influenza viruses A H1N1 and A H3N2 and as well as A H1N1pdm09 subtypes of influenza A and influenza B viruses have been reported to circulate in humans (Mmbaga *et al*., 2012). Influenza A H1N1 has been reported to infect swine while influenza A H6N2, H7N3, H2N4, H3N2, H5N2, H5N1 and H6N4 subtypes have been reported to infect birds (Brown *et al.*, 2009; Hutchinson and Yamauchi, 2018) (Figure 4).

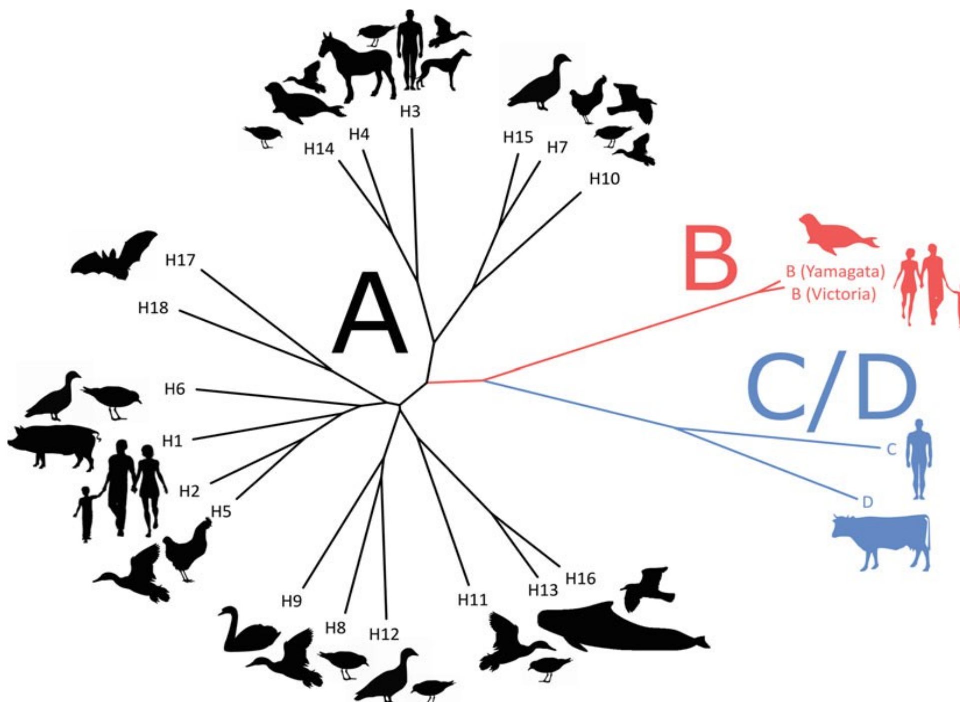

Figure 4: Distribution of influenza A subtypes and influenza B, C and D among different species. Source: (Hutchinson and Yamauchi, 2018).

2.3.2 Transmission

Influenza, like other respiratory viruses spread via contact or aerosol routes. Contact transmission can be through direct (contaminated hands) or indirect (fomites) virus transfer from infected to susceptible person (Krutter *et al.*, 2018).

Transmission of viruses through air can occur via droplets or aerosols. Transmissions of viruses depend on environmental factors e.g. humidity, temperature, crowding conditions, but also on host factors such as receptor distribution throughout the respiratory tract (Krutter *et al.*, 2018).

2.3.3 Host range

Influenza A and B are of the public health importance. All known influenza A virus subtypes can infect birds and only two influenza A virus subtypes (i.e., H1N1 and H3N2) are currently in general circulation among people (Hutchinson and Yamauchi, 2018). Some subtypes are found in other infected animal species. For example, H7N7 and H3N8 virus infections can cause illness in horses and H3N8 in horses and dogs while swine triple reassortant (tr) H1N1, trH3N2 virus and trH1N2 influenza virus causes infections in pigs (CDC, 2017).

2.4 Diagnosis of Influenza

2.4.1 Clinical symptoms

Influenza infection in adults is characterized by fever, muscle pain and headaches (Hutchinson and Yamauchi, 2018). About one-third of infants with respiratory viral infections develop lower respiratory tract symptoms such as tachypnoea, wheeze, severe

cough, breathlessness and respiratory distress, nasal flaring, thoracic in-drawings and crepitation while the upper viral respiratory tract infection leading to symptoms like common cold, coryza, fever, cough and may lead to lethargy and poor feeding (Tregoning and Schwarze, 2010).

2.4.2 Laboratory diagnosis

2.4.2.1 Viral detection

Both conventional RT-PCR and real time RT-PCR can be used to detect influenza viruses (Bulimo *et al.*, 2012; Kim and Barun, 2012). One step RT-PCR involves the use of oligo-dT or random primers for reverse transcription and in two steps RT-PCR reverses transcription is performed and then PCR is carried out to amplify synthesized cDNA in the same reaction. Briefly, RNA extracted from the influenza sample is purified and transcribed using the oligonucleotides specific to the target sequence producing cDNA which is amplified during PCR reaction. Viral culture is another technique used to detect influenza viruses in which the viruses are grown in the laboratory using different cell cultures such as monkey kidney cells, Madin Darby canine kidney (MDCK) cells and A549 cells. Cells are used to detect influenza viruses following cytopathic effect caused by virus after inoculation. The method takes almost 2 weeks to get the results (Kim and Barun, 2012).

Heamagglution test is used to detect the level of influenza present in a sample. Briefly, a serial dilution of the virus is prepared mixed in a well of the tray and a specific amount of red blood cells is added. Viral heamagglutinin proteins binds to sialic acid receptors on the erythrocytes causing the formation of a lattice that coat the wells, this property is called hemagglutination. The red blood cells that are not bound by influenza virus

precipitate down by gravity to the bottom of a well and form a button, Amino acid substitutions in or around the receptor binding site of the HA molecule of influenza viruses affect the capacity of the virus to agglutinate red blood cells (Van Baalen *et al.,* 2014).

2.4.2.2 Serological tests

The enzyme-linked immunosorbent assay (ELISA) is an immunological test commonly used to measure antibodies produced in the body following infection in biological samples. The plates are coated with primary antibodies and sample are added and incubated. Any antigen found in the sample will bind to the capture antibody already coating the plate. The detected antibody are then added, labelled with an enzyme usually horse radish peroxidase or alkaline phosphatase. Finally, a substrate is added to the plate and a coloured product which is produced in a positive sample can be measured using a plate reader (Van Baalen *et al.,* 2014). Immunofluorescence assay detects and visualize the viral proteins expressed in cells via antigen antibody reaction, it involves grown of the influenza infected cells on cover glass and fixed them with formaldehyde. The cells are then exposed to specific primary antibodies. Then, the secondary antibody which is specific to Fc fragment of IgG molecule of the primary antibody is applied which is linked to fluorescence dye. The antigens are then visualized under a fluorescence microscope.

2.5 Management of Influenza Respiratory Infections

2.5.1 Vaccination

Influenza vaccine is used to stimulate production of antibodies against influenza virus infection. There are several influenza vaccines that can be used which include

quadrivalent vaccine which is used to prevent against influenza A (H1N1) virus, influenza A (H3N2) virus, and two influenza B viruses. Trivalent vaccine for influenza A (H1N1) virus, an influenza A (H3N2) virus and one influenza B Victoria lineage virus and is the current used vaccine for influenza virus having A/Brisbane/02/2018(H1N1) pdm09, A/Kansas/14/2017(H3N2) and B/Colorado/06/2017 Victoria linage (CDC 2019).

2.5.2 Treatment of influenza

Many people will recover with taking of plenty fluids and resting. Antiviral medications are used in severe or prolonged viral respiratory infections and most of them are inhibited to infants. Currently treatment for severe viral respiratory infection in infants relies on supportive measures such as supplementation of oxygen, monitoring of apnoea, nasogastric tube feeding or intravenous fluids and mechanical ventilation (Tregoning and Schwarze, 2010). Sialic acid analogues, notably oseltamivir and zanamivir act as neuraminidase inhibitors and are the main class of drug licensed to treat influenza infections (CDC, 2017). They can also be used for chemoprophylaxis among individuals one year and older in cases of outbreaks and exposure in high-risk groups (Hutchinson and Yamauchi, 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

This was a hospital-based study that involved samples collected from Morogoro, Mbeya and Zanzibar in Tanzania. Morogoro region occupies a total area of 72 939 square kilometres with an estimate total population of 2 218 492 people (URT, 2012). Mbeya region covers an area of 63 420 square kilometres and is allocated at latitudes 7° and 9° 31” South of Equator and Longitudes 32° 32$^{''}$35$^{''}$ East of Greenwich with elevation of 475 meters above sea level. Zanzibar covers a total area of 2 461 kilometres square and is consisting of many small islands and two large ones Unguja and Pemba Island. In each region, only one hospital was chosen for sample collection. Mbalizi hospital from Mbeya, Mzinga hospital from Morogoro and Bububu hospital from Zanzibar (Figure 5).

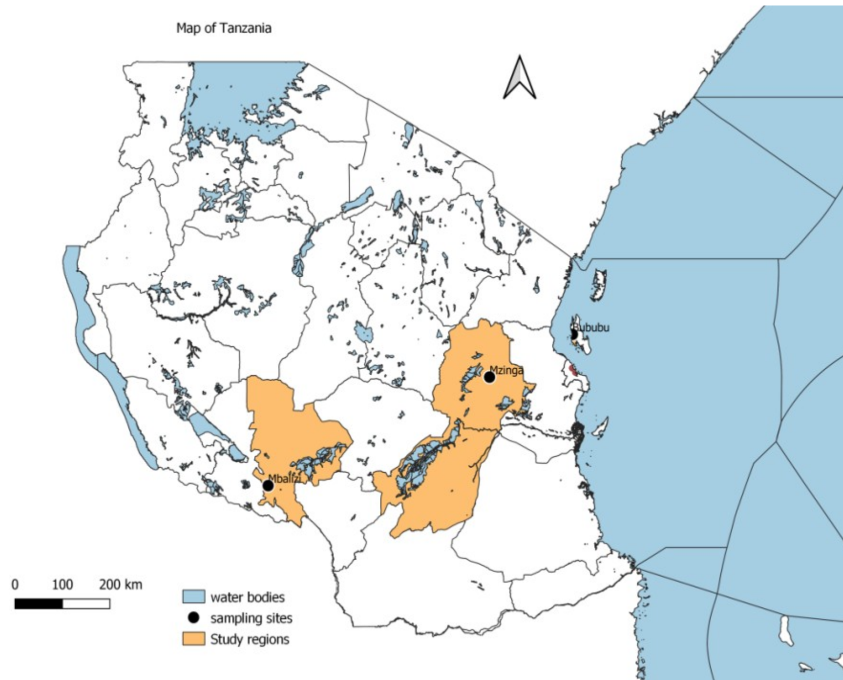

Figure 5: Tanzania map showing locations where sampling was conducted

3.2 Sample Size

The sample size was calculated by using the following formula (Martin *et al*., 1987).

$$N=\frac{Z^2p\ (1.0-p)}{d^2}$$

Where N = sample size

Z = standard normal deviate (1.96= 95% confidence level)

p = proportion in the target population estimate = 8% Mmbaga *et al*. (2012)

d = degree of accuracy desired, set at 5% or 0.05

$$N = \frac{(1.96)^2 X\ 0.08\ X\ (1.0- 0.08)}{(0.05)^2} = 113$$

According to this formula the minimum sample size is 113 samples

3.3 Study Design

This was a retrospective cross sectional study using archived samples of nasopharyngeal and oropharyngeal swabs collected during January to December 2019. The samples were collected purposively after meeting the case definition for influenza-like illness among outpatients seeking care from hospitals in Morogoro, Mbeya and Zanzibar. The samples were collected from individual of all age groups with fever ≥ 38 ºC and cough or sore throat in the absence of other diagnoses.

3.4 Laboratory Procedures

3.4.1 Sample preparation

The oropharyngeal and nasopharyngeal swabs were stored in viral transport media and transported to the virology laboratory. In the laboratory, samples were stored at -80ºC refrigerator and they were handled in biological safety cabinet class II. A total of 100 µL aliquot was used for RNA extraction.

3.4.2 RNA extraction

The viral RNA template was extracted using Zymo RNA extraction kit (Zymo research, CA, USA), according to manufacturer’s instruction. Briefly, 100 µL of sample was mixed with 300 µL of viral RNA lysis buffer and incubated at room temperature (15–25°C) for 10 min. Afterward, 400 µL of a mixture were applied to the Zymo spin column then centrifuged at 10 000 rpm for 2 min and the filtrate was discarded. Some 500 µL of viral wash buffer was added in a Zymo spin Column and centrifuged at 10 000 rpm for 2 minutes to remove PCR inhibitor and the filtrate was discarded. Then, 40 µL of DNase/RNase free water was added and incubated at room temperature for 1 minute for elution and centrifuge at 10 000 rpm for 1 minute for collection of viral RNA in

microcentrifuge tube as a template of RT-PCR. Positive and negative control were included in RNA extraction.

3.4.3 Primer reconstitution

Primers were reconstituted at 100 µM by adding 880 µM of nuclease free water to the tube containing 88 nM lyophilized primer powder. The prepared stock primers at 100 µM were labelled and stored at -80ºC until reconstituted to 10 µM working solution. The working primer solution of 10 µM was prepared by adding 90 µL of nuclease free water in a tube containing 10 µM of the stock primer. The prepared working primer solution was then labelled and used for RT-PCR.

3.4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Hemagglutinin genes were targeted by RT-PCR assay for detection of influenza viruses using the specific primers. The sequences of the three sets of primers used for gene amplification are indicated in Table 1.

Table 1: Primer sequence used for detection of influenza A and B

Gene	Primer name	Primer sequence (5’ to 3’)	Amplicon size	Target virus	Reference
HA	FluAHA3F FluAHA3R	AAA TAT GCG ACA GTC CTC A CAT TAT TGA GCT TTT CCC ACT TC	650 bp	influenza A H3N2	Matyushenko *et al.*, 2017)
HA	FluAHA1F FluAHA1R	CGG GAA ACT ATG CAA ACT AAG AGG GCA ATC GTG GAC TGG TCT ATC TG	700 bp	influenza A H1N1pdm	Matyushenko *et al.*, 2017)
HA	FluBHAF FluBHAR	GAA GGA TGG GAA GGA ATG AT TCT GGT TGC ATT TGT GTT TGG TTT	410 bp	influenza B	Matyushenko *et al.*, 2017)

Amplification reactions for detection of Influenza A and Influenza B was performed separately in a 25 µL mixture containing 22.4 µL ready to go master mix and 2.6 µL of RNA template. Master mix used contained 0.7 µL of each forward and reverse primer, 1 µL of RT-PCR enzyme, 7.5 µL of molecular grade water and 12.5 µL of reaction buffer. A positive and negative control was included in each assay. Composition of master mix reagents is shown in Table 2.

Table 2: Composition of master mixture for one step RT-PCR for the detection of influenza A and B

Reagent	**Volume (µL)**
2X RT-PCR buffer	12.5
Forward primer	0.7
Reverse primer	0.7
RT-PCR Enzyme mix	1
Molecular grade water	7.5
Total volume	22.4

The cycling parameters for RT-PCR were 50°C for 20 min and 94°C for 15 min (RT), followed by 35 cycles of 95°C for 15 seconds, 52°C for 30 seconds, 72°C for 1 minute and 1 cycle of 72°C for 10 minutes for amplification of cDNA. Separation and visualization of PCR products was done in agarose gel electrophoresis. Briefly, 5µL aliquots of each amplification were subjected to electrophoresis in 1% agarose gel stained with 4 µL of Gel Red. 100 bp DNA ladder was used to determine the size of PCR products and detection was confirmed by comparison to PCR products generated from wild-type strain which was used as positive controls in all PCR analysis. In order to ensure specific product amplification, negative controls (reactions with no DNA templates) was included in all PCR analyses.

3.4.5 Sequencing

Four representatives PCR amplicons were randomly selected for sequencing using Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA). The FluAHA1F, FluAHA1R, FluBHAF and FluBHAR primers were used to sequencing the isolates. The forward and reverse complement nucleotides sequences delimited by forward and reverse primers of structural hemagglutinin region PCR products of influenza virus were assembled, and edited to obtain a reliable consensus sequence.

3.5 Data Management and Analysis

Data were populated in an Excel file and analysis was carried out using R software by computing *P*-values and proportions of respiratory tract infections due to viral aetiology. The obtained nucleotide sequence of influenza viruses was subjected to NCBI using a BLASTN search program (NCBI~hppt://.www.NCBI.nlm.nih.gov) to determine identity with other published influenza strains available in PubMed. The nucleotide sequence was aligned using Clustal Wallis in MEGA X software. The phylogenetic analysis was done using the Maximum Likelihood method (Kimura, 1980), utilizing Kimura 2-parameter model included in MEGA X (Kumar *et al.,* 2018).The phylogenetic tree was generated based on the nucleotide sequences of HA gene, which involved 4 nucleotide sequences form the samples and 41 nucleotide sequences from references strains obtained from the Genbank. The topology of the tree was estimated by the bootstrap method of analysis of 1000 replicates.

3.6 Ethical Considerations

This study received ethical approval from the Medical Research Committee of the Tanzania National Institute for Medical Research (Ref. No. NIMR/HQ/R.8a/ Vol.1/1112).

CHAPTER FOUR

4.0 RESULTS

4.1 Socio-demographic Characteristics

A total of 735 subjects were recruited into this study. Of these, 309 were from Zanzibar, 316 from Morogoro and 110 from Mbeya region. Among the subjects, 356 (48%) were males and 379 (52%) were females (Table 3).

4.2 Prevalence of Influenza Virus

The total of 93 samples were RT-PCR positive for influenza virus showed a specific band of 650 bp for H3N2, 700 bp for H1N1pdm and 410 bp for influenza B virus. The electrophoresis gel image indicated that there was intact DNA that was suitable for downstream nucleotide sequencing (Figure 6).

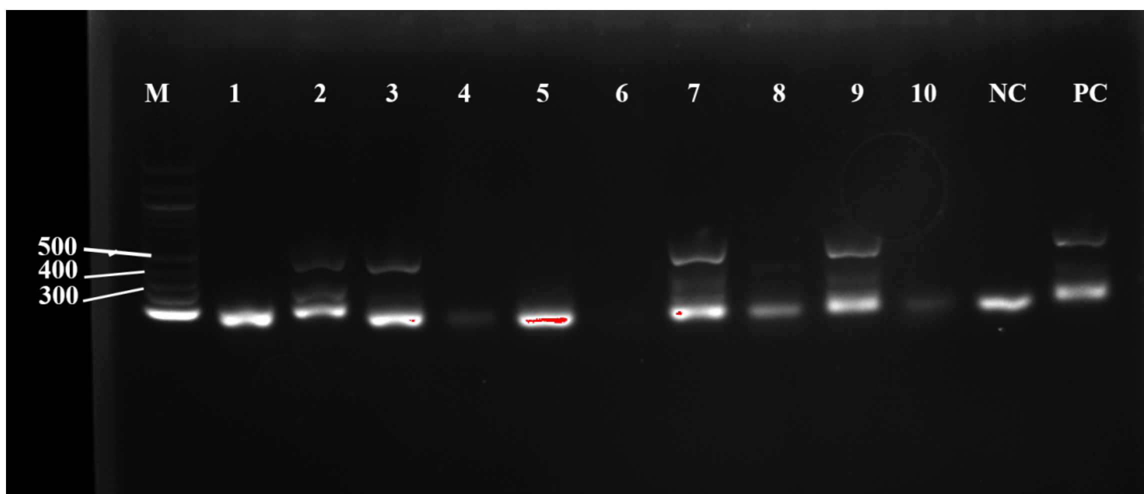

Figure 6: Gel picture showing band for positive sample at 410 bp for influenza B virus. Lane M, 100-bp DNA ladder; Lanes 1–10, tested samples identification numbers; Lane PC, Known positive control, Lane NC, negative control and Lane 2, 3, 7 and 8 are positive tested sample.

Among 735 samples tested, 93 (13%) were found positive for influenza virus. The prevalence of respiratory viral infection in males was 11.5% (41/356) while in females was 13.7% (52/379). The influenza virus positive rate appeared to vary with age. The higher positive rate of influenza was observed in the age groups of 0-9 and 20-34 years. The lowest positive rate was observed in the age group of >35 years old. The prevalence of influenza infection varied between different sites. It was significantly higher in Zanzibar (16.8%; 52/306), than in Morogoro (9.1%; 10/110) and Mbeya (8.2%; 26/316) ($\chi2 = 10.57$ $p<0.05$) (Table 3).

Table 3: Demographics characteristics of patients tested for influenza viruses in 2019

Characteristics	Overall	Controls	Cases	Inf A	Inf B	$\chi 2$	P-value
N	735	642 (87)	93 (13)	55 (59)	38 (41)		
Sex						0.62	0.43
Female	379 (52)	327 (51)	52 (56)	32 (58)	21 (55)		
Male	356 (48)	315 (19)	41 (44)	24 (42)	17 (45)		
Sites						10.57	<0.05
Zanzibar	309 (42)	257 (40)	52 (56)	32 (58)	20 (53)		
Mbeya	316 (13)	290 (35)	26 (28)	13 (24)	13 (34)		
Morogoro	110 (15)	95 (15)	15 (16)	10 (18)	5 (13)		
Age (years)						12.82	0.38
0-4	104 (16)	95 (15)	9 (10)	6 (11)	3 (8)		
5-9	58 (10)	48 (7)	10 (11)	4 (7)	6 (16)		
10-14	34 (5)	30 (5)	4 (4)	3 (5)	1 (3)		
15-19	37 (6)	31 (5)	6 (6)	4 (7)	2 (5)		
20-24	106 (17)	91 (14)	15 (16)	10 (18)	5 (13)		
25-29	87 (14)	77 (12)	10 (11)	3 (5)	7 (18)		
30-34	109 (17)	93 (14)	16 (17)	10 (18)	6 (16)		
35-39	47 (7)	43 (7)	4 (4)	1 (2)	3 (8)		
40-44	55 (9)	53 (8)	2 (2)	1 (2)	1 (3)		
45-49	22 (3)	17 (3)	5 (5)	2 (4)	3 (8)		
50-54	14 (2)	12 (2)	2 (2)	1 (2)	1 (3)		
55-59	16 (2)	12 (2)	4 (4)	4 (7)	0 (0)		
60+	46 (7)	40 (6)	6 (6)	6 (11)	0 (0)		
Months						67.9	<0.05
Jan	50 (8)	38 (6)	12 (13)	11 (20)	1 (3)		
Feb	67 (10)	63 (10)	4 (4)	4 (7)	0 (0)		
Mar	66 (10)	48 (7)	18 (19)	3 (5)	15 (39)		
Apr	46 (7)	38 (6)	8 (8)	4 (7)	4 (11)		
May	55 (9)	41 (6)	14 (15)	10 (18)	4 (11)		
Jun	44 (7)	41 (6)	3 (3)	1 (2)	2 (5)		
Jul	52 (8)	51 (8)	1 (1)	1 (2)	0 (0)		
Aug	81 (13)	79 (12)	2 (2)	1 (2)	1 (3)		

Sep	72 (11)	70 (11)	2 (2)	2 (4)	0 (0)
Oct	86 (13)	83 (13)	3 (3)	2 (4)	1 (3)
Nov	54 (8)	41 (6)	13 (14)	4 (7)	9 (24)
Dec	62 (10)	49 (7)	13 (14)	12 (22)	1 (3)

4.3 Seasonal Variation of Influenza in a Year

In 2019, influenza viruses were detected throughout the year though the prevalence varied with time. Generally, the peak influenza activity occurred during November to January and March to May and the low influenza activity was observed from June to October. There was a significant difference between the monthly prevalence ($\chi2 = 67.9$ $p<0.05$) (Figure 7).

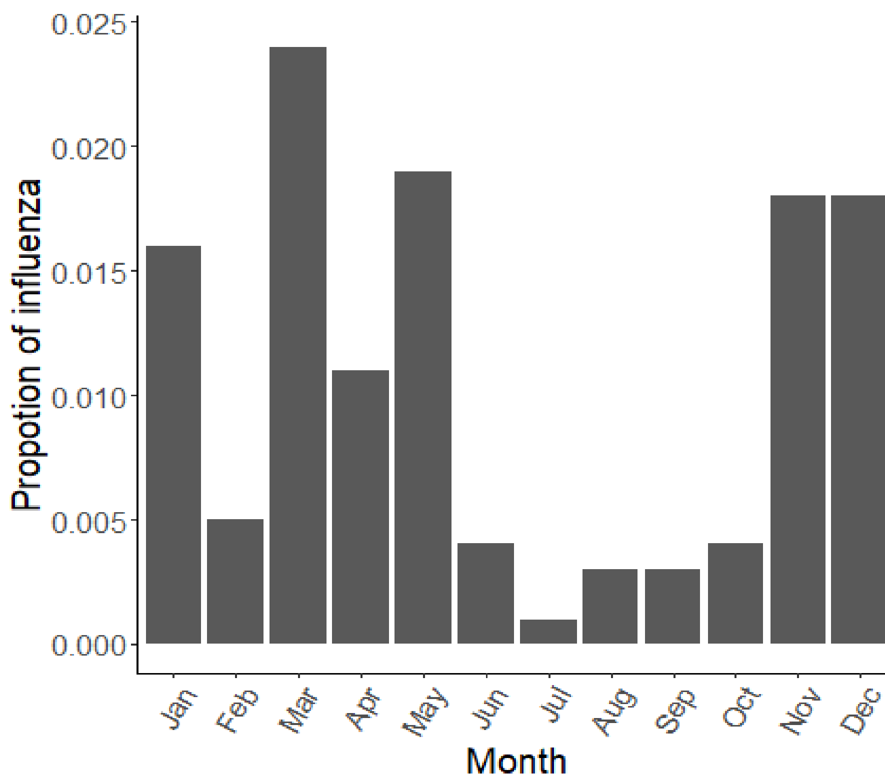

Figure 7: Monthly patterns of the prevalence of influenza virus, 2019

The prevalence of influenza A (55/93; 59%) virus was higher than that of Influenza B (38/93; 41%). Specifically, the prevalence of Influenza A subtype H3N2 was relatively higher (31/55; 56%) than influenza A H1N1pdm09 (21/52; 38%). Both influenza B and seasonal influenza A H3N2 and H1N1 pdm09 were detected in high peaks during May (Figure 8).

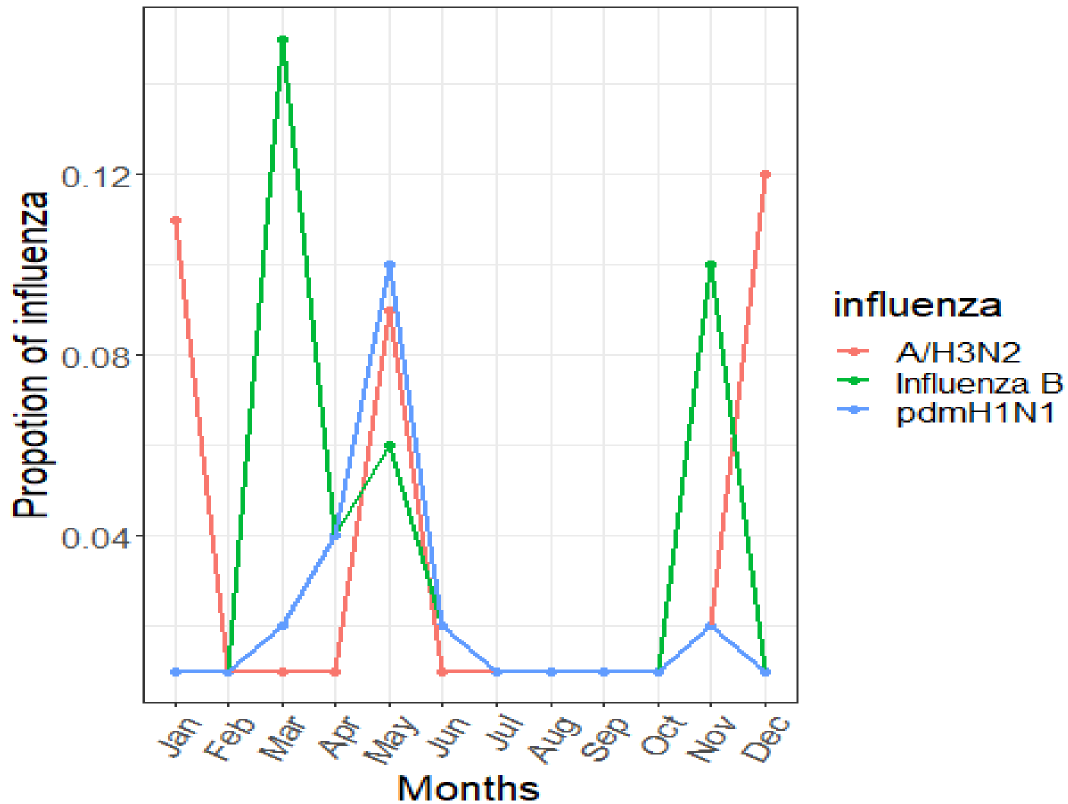

Figure 8: Monthly distribution of influenza A subtypes (H3N2 and H1N1pdm) and influenza B type by month in 2019

The detected influenza A subtypes and influenza B viruses did not have statistical significant difference in the distribution between the age groups ($\chi=212.82$ $p>0.05$). The prevalence of Influenza A H3N2 subtype (8%) and influenza B (10%) were higher in lower age group 0-9, 20 - 24 and 30-34 years age categories. Influenza A/H3N2 virus was predominant (8%) in samples collected from those 55 years old and above (Figure 9).

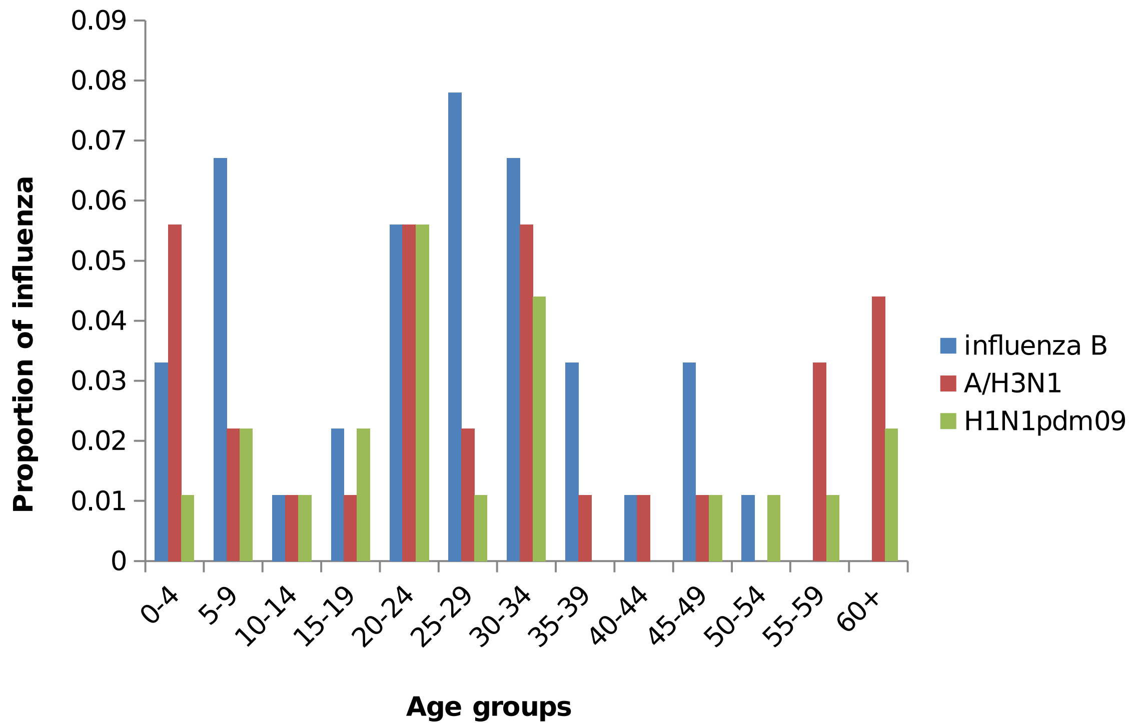

Figure 9: Distribution of detected influenza A subtypes and influenza B by age group

4.4 Phylogenetic Analysis

The sequences obtained from this study were assigned with the names; A/Tanzania/BMH1674/2019, B/Tanzania/BMH930/2019, B/Tanzania/BMH0861/2019 and B/Tanzania/MZC1378/2019, based on the convention for nomenclature of influenza viruses. The HA gene nucleotide sequence of A/Tanzania/BMH1674/2019 (from Zanzibar) was 712 bp long, while that of B/Tanzania/BMH930/2019 (from Zanzibar), B/Tanzania/BMH0861/2019 (form Zanzibar) and B/Tanzania/MZC1378/2019 (from Morogoro) were 409 bp long. A BLAST search using NCBI database for HA nucleotide sequences revealed the maximum sequence identity of 99.86% of A/Tanzania/BMH1674/2019 isolate with A/Utah/24/2019 strain from United State and 95% identity with A/Kenya/265/2013 isolate from Kenya.

All the three HA nucleotide sequences of influenza B virus B/Tanzania/BMH930/2019, B/Tanzania/BMH0861/2019 and B/Tanzania/MZC1378/2019 had maximum nucleotide identity of 100% among one another, 99.76% similar with B/Singapore/G2-15.1/2014 isolate from Singapore, 99% similar with B/Kenya/2050/2010 isolate from Kenya and 95% similar with B/Uganda/MUWR-154/2010 isolate from Uganda.

Nucleotide sequence alignment of B/Tanzania/BMH930/2019, B/Tanzania/BMH0861/2019 and B/Tanzania/MZC1378/2019 sequence with influenza B HA nucleotide sequences of selected reference strains from Genbank was done using Clustal Wallis in MEGA X software. The nucleotide sequences were then translated and align. The alignments of amino acid reveal that all isolates from this study had similar amino acid alignment with other isolates of selected reference strains from Genbank. (Figure 10 and 11).

Strain	Sequence
1. B/Tanzania/BMH930/2019	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
2. B/Tanzania/BMH0861/2019	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
3. B/Tanzania/MZC1378/2019	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
4. B/Singapore/G2-15.1/2014	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
5. B/Singapore/G2-7.1/2013	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
6. B/New York/06/2020	? G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
7. B/India/Sri-121249/2011	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
8. B/Alaska/03/2013	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
9. B/California/12/2015	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
10. B/Florida/4/2006	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
11. B/Massachusetts/02/2012	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
12. B/Johannesburg/20/2008	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
13. B/Algeria/G485P0/2009	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
14. B/Cameroon/10V-2035/2010	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
15. B/Nairobi/15/2008	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
16. B/Brisbane/60/2008	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
17. B/China/b151/2016	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
18. B/Kenya/2050/2010	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T Q
19. B/Uganda/MUWR-154/2010	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K
20. B/Madagascar/7766/2010	E G G W E G M I A G W H G Y T S H G A H G V A V A A D L K S T Q E A I N K I T K

Figure 10: Amino acid sequence alignment of influenza B virus Tanzanian isolates from this study with the reference selected strain from the Genbank.

Figure 11: Amino acid sequence alignment of influenza A virus Tanzanian isolates from this study with the reference selected strain from the Genbank.

Phylogenetic analysis shows that A/Tanzania/BMH1674/2019 clustered with A/Colorado/9776/2019, A/Utah/24/2019 and A/Texas/109/2019 isolate from United States in phylogenetic tree with the maximum likelihood method (Figure 12). The B/Tanzania/BMH930/2019, B/Tanzania/BMH0861/2019 and B/Tanzania/MZC1378/201 isolates were grouped in one cluster in phylogenetic tree with the maximum likelihood method. All influenza B isolates were Victoria lineage as they were all clustered with B/Brisbane/60/2008 Victoria strain (Figure 13). The accession numbers used in tree construction of influenza A and B are summarized in Table 4 and 5, respectively.

Table 4: Summary of influenza virus isolates H1N1pdm09 subtype used in construction of phylogenetic tree

Isolate	Country	Host species	Year	Accession number
A/Utah/24/2019	United state	human	2019	MN004554.1
A/Texas/109/2019	United States	human	2019	MK856197
A/Tanzania/BMH1674/2019	Tanzania	human	2019	This study
A/South Korea/7602/2018	Korea	human	2018	MK380756.1
A/Rabat/RS113/2009	Morocco	human	2009	JX204761.1
A/Colorado/9776/2019	United States	human	2019	MT639657.1
A/Italy/9228/2019	Rome	human	2019	MT638114.1
A/Florida/70/2019	United States	human	2019	MN229895.1
A/California/150/2019	United States	human	2019	MN229815.1
A/Rabat/HR1230/2010	Morocco	human	2010	JX204762.1
A/Rabat/044/2011	Morocco	human	2011	JQ319710.1
A/Casablanca/60/2011	Morocco	human	2011	CY099757.1
A/Casablanca/92/2011	Morocco	human	2011	CY099768.1
A/Kenya/267/2013	Kenya	human	2013	KF451913.1
A/Kenya/265/2013	Kenya	human	2013	KF451903.1
A/Egypt/42/2014	Egypt	human	2014	KP702181.1
A/Addis Ababa/WR2848T/2009	Ethiopia	human	2009	CY071826.2
A/Kenya/138/2011	Kenya	human	2011	JQ396235.1
A/Kenya/151/2011	Kenya	human	2011	JQ396244.1
A/Tunisia/422/2011	Tunisia	human	2011	CY080590.1

Table 5: Summary of influenza B virus isolates used in construction of phylogenetic tree

Isolate	Country	Host	Year	Genbank accession
B/Tanzania/BMH930/2019	Tanzania	human	2019	This study
B/Tanzania/BMH0861/2019	Tanzania	human	2019	This study
B/Tanzania/MZC1378/2019	Tanzania	human	2019	This study
B/Singapore/G2-15.1/2014	Singapore	human	2014	MN483445.1

B/Singapore/G2-7.1/2013	Singapore	human	2013	MN480787.1
B/New York/06/2020	New York	human	2020	MT243491.1
B/India/Sri-121249/2011	India	human	2011	MH703066.1
B/Alaska/03/2013	USA	human	2013	KF216476.1
B/California/12/2015	USA	human	2015	KY116926.1
B/Florida/4/2006	USA	human	2006	EU515992.1
B/Brisbane/3/2007	USA	human	2007	KP460690.1
B/Massachusetts/02/2012	USA	human	2017	KC892118.1
B/Madagascar/7766/2010	Madagascar	human	2009	NA
B/Nairobi/15/2008	Kenya	human	2008	GQ456063.1
B/Cameroon/10v-2035/2010	Cameroon	human	2010	NA
B/Uganda/MUWR-154/2010	Uganda	human	2010	NA
B/Algeria/G485p0/2009	Algeria	human	2009	NA
B/China/b151/2016	China	human	2016	MN795818.1
B/Kenya/2050/2010	Kenya	human	2010	NA
B/Johannesburg/20/2008	South Africa	human	2008	NA

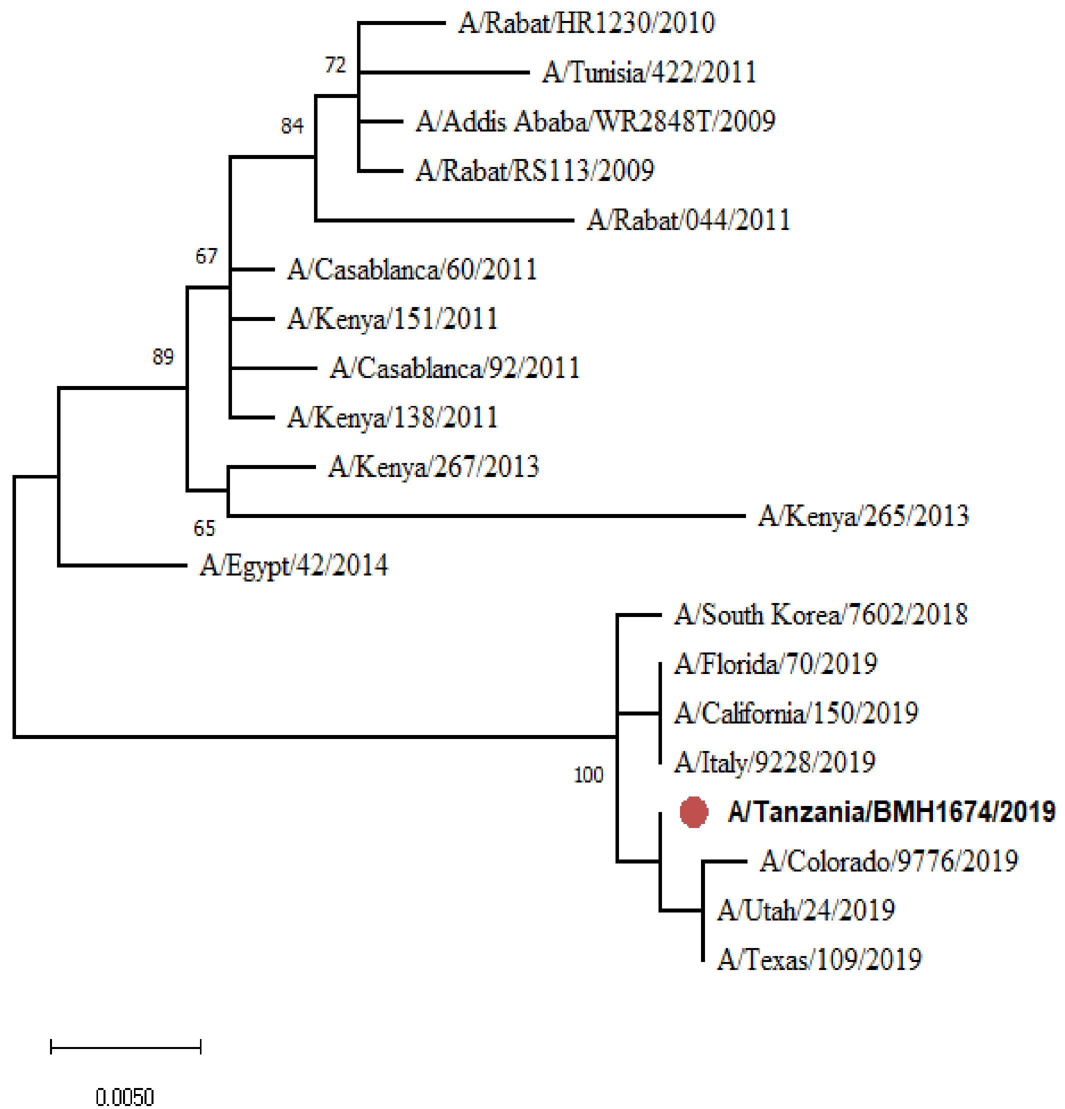

Figure 12: Phylogenetic tree of the hemagglutinin (HA) gene segment of the Tanzanian influenza A (H1N1) pdm09 virus (in bold font) and selected reference strains at the nucleotide level.

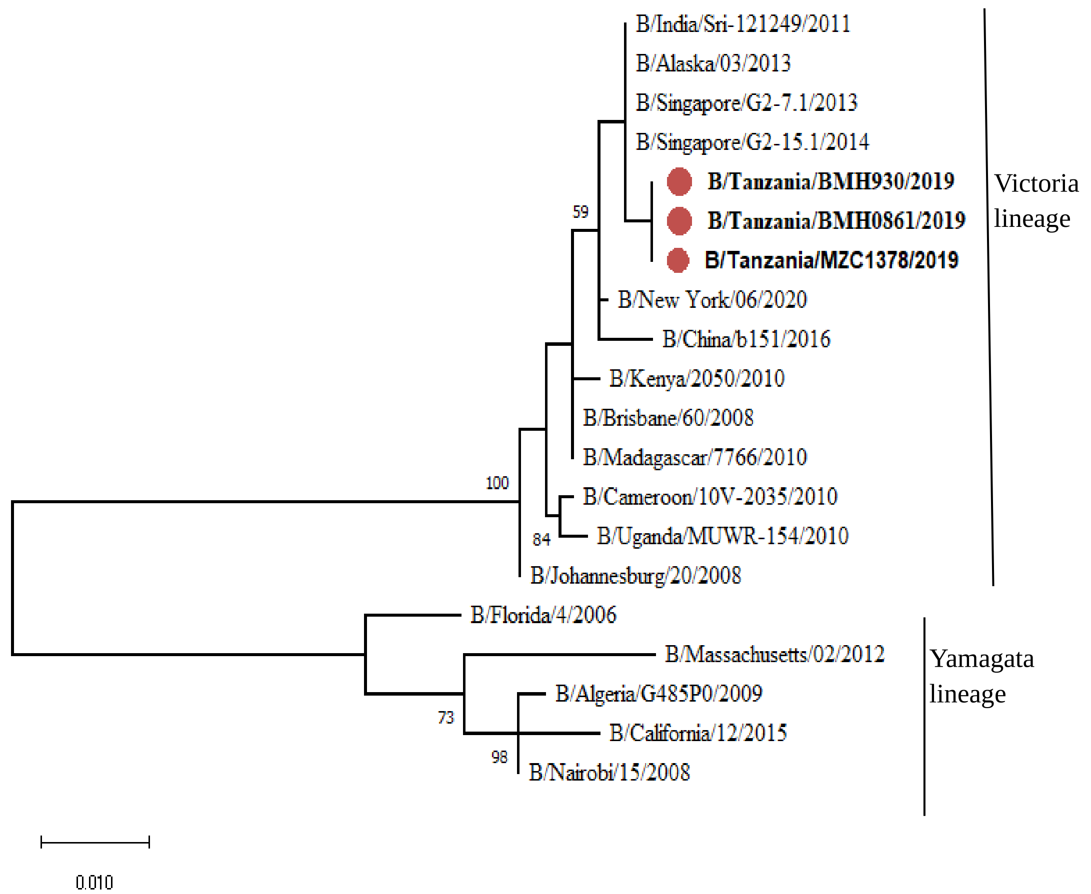

Figure 13: Phylogenetic tree of the hemagglutinin (HA) gene segment of the Tanzanian influenza B viruses (in bold font) and selected reference strains at the nucleotide level.

CHAPTER FIVE

5.0 DISCUSSION

This study aimed at determining the prevalence and genetic characteristics of influenza virus circulating in human populations in Tanzania. The present study confirmed existence of influenza viruses among outpatients presenting with influenza like illness signs in selected hospitals of Tanzania. The investigation revealed 13% of the tested subjects to be positive by RT-PCR targeting influenza viruses, where influenza A H1N1pdm09, influenza A H3N2 and influenza B viruses were found in the tested samples. This prevalent of influenza detected is slightly higher compared to 8% prevalence earlier reported in Tanzania (Mmbaga *et al.*, 2012). About one-fifth of the positive cases were seen in children below nine years of age. In a study in Kenya, a much lower prevalence (7%) among children of the same age (McMorrow *et al.*, 2015). This is also supported with the report from Kishamawe and colleagues (2018) where they observed that, most of the deaths due to respiratory diseases in Tanzania occur among children below five years. Respiratory diseases are known to affect largely children less than five years (Bourgeois *et al.*, 2009; Kurskaya *et al.*, 2017, McMorrow *et al.,* 2015). Respiratory infections have high health impacts in children as their immunity have not adapted to the surrounding environment. In sub-Saharan Africa countries prevalence of influenza virus varies from 5.1%–25.9% (McMorrow *et al.,* 2015).

This study has identified influenza A H1N1pdm09, influenza A H3N2 and influenza B viruses to be prevalent in Tanzania. The predominance of Influenza A H3N2 subtype has also been reported in in Kenya (McMorrow *et al.,* 2015) and Burkina Faso (Sanou *et al.,* 2018). Like in our study, influenza A H1N1pdm09 and influenza A H3N2 have been

found in among children in Russia (Kurskaya *et al.,* 2018). The relatedness between isolates obtained in this study with isolates from other Sub Saharan Africa countries provide evidence that distribution of influenza is not limited to certain geographical locations.

Tanzania is in the tropical region where influenza epidemics occur throughout the year, different from temperate climates where seasonal epidemics occur mainly during winter season (WHO, 2018). The first peak for both influenza B and influenza A was observed from March to May, the same observation was reported by Mmbaga and colleagues (2012) in the study involving sentinel surveillance for influenza in Tanzania. Another peak for influenza activity was noted from November to January. The two observed peak of influenza disease might be influenced by rainy condition, the first long rains start from March to May and short rains start from November to December. Low influenza activity was observed from June to October which is the long dry season in Tanzania.

The strains that clustered in the same group in the phylogenetic tree are genetically closely related and might have the same ancestral origin. And this is important in molecular epidemiology as it help to know genotype that will give the source of origin, transmission and migration history. From this study, four influenza isolate were included in phylogenetic analysis. The BLAST search of A/Tanzania/BMH1674/2019 isolate showed the highest nucleotide similarity with A/Utah/24/2019 and A/Texas/109/2019 strain from Texas in United States. The isolate also had 95% nucleotide similarity with A/Kenya/265/2013 isolate from Kenya. The BLAST search of B/Tanzania/BM H930/2019, B/Tanzania/BMH0861/2019 and B/Tanzania/MZC1378/2019 reveal the nucleotide sequence similarities with B/Singapore/G2-15.1/2014 isolate from Asia, B/Kenya/2050/2010 isolate from Kenya and B/Uganda/MUWR-154/2010 isolate from

Uganda. Genetic similarity among isolates from this study confirmed that the isolates were from the single influenza outbreak. Genetic relatedness of Tanzanian strains to those from other part of the world provide further evidence that distribution of influenza is not limited to certain geographical locations and there is a possibility of viruses to be introduced into Tanzania from other neighbouring countries.

Phylogenetic analysis revealed the isolates from our study showed low genetic diversity and that all influenza B isolates were Victoria lineage as they were all clustered with B/Brisbane/60/2008-like (CDC, 2010). Most of the isolates in our study shared high similarity with characterized reference strains from the neighbouring countries and from other part of the word. This indicates that the Tanzanian influenza viruses could be because of the introduction of new viruses into Tanzania from the rest of the world rather than independent evolution of variants within the country.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study has shown that influenza A H1N1pdm09 and H3N2 subtypes are prevalent in Tanzania and that children below 9 years old are the most affected. Also, the detected influenza isolates were genetically similar to the isolates detected from other parts of the world including Kenya and Uganda.

6.2 Recommendations

From the present results, it is recommended that there need to strengthen influenza surveillance programme covering all regions of Tanzania to provide evidence of the burden, distribution and pattern in the country. Also education of self-precaution measures such as wearing a mask, keeping physical distance, avoiding crowds, washing hands and cough into a bend elbow or tissue should be provided to the society in order to prevent or minimise the health effects due to respiratory infections.

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