Molecular Detection and Genetic Assessment of Influenza Viruses in Islamabad

By

Asim Saeed

Registration no: 02272111011

Department of Biotechnology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad

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By

Asim Saeed

Supervised by

Dr. Muhammad Ali

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In the name of Allah, The Most Gracious, The Most Merciful

Certificate of Approval

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan accepts the dissertation entitled **"Molecular Detection and Genetic Assessment of Influenza Viruses in Islamabad"** submitted by **Mr. Asim Saeed** in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in **Biotechnology.**

Supervisor _____

Dr. Muhammad Ali
Assistant Professor
Department of Biotechnology
Quaid-i-Azam University, Islamabad

External Examiner _____

Dr. Bushra Uzair
Assistant Professor
Department of Microbiology
International Islamic University Islamabad

Chairperson _____

Dr. Javaria Qazi
Associate Professor
Department of Biotechnology
Quaid-i-Azam University Islamabad

Dated **25-05-2023**

DECLARATION OF ORIGINALITY

I hereby declare that the work **"*Molecular Detection and Genetic Assessment of Influenza Viruses in Islamabad"*** accomplished in this thesis is the result of my own research carried out in ***Infectious Diseases and Molecular Pathology laboratory***, Department of Biotechnology, Quaid-I-Azam University, Islamabad, Pakistan. This research study has not been published previously nor does it contain any material from the published resources that can be considered as a violation of international copyright law. Furthermore, I also declare that I am aware of the term ~~–~~copy right" and plagiarism. If, any copyright violation is found in this research work, I will be responsible for the consequence of any such violation.

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Asim Saeed

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I dedicated this work, especially to my brothers "Imran Saeed & Sohrab Saeed", my Parents, and my charming family

Thank you for your being

My best friends,

My comfort when things go wrong,

The ones who encourage me, believe me,

Change my "weak" to strong,

And for filling my heart with such a pride

I feel like the happiest person alive

With you all by my side

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List of Abbreviations

IAV	Influenza A virus
IBV	Influenza B virus
ICV	Influenza C virus
IDV	Influenza D virus
NA	Neuraminidase
HA	Hemagglutinin
M	Matrix
PB	Basic polymerase
PA	Acidic polymerase
NP	Nucleoprotein
WHO	World health organization
CDC	Centre for disease control and prevention
NIH	National institute of health
U.S.	United states
RNA	Ribonucleic acid
nRNA	Messenger RNA
cRNA	Complementary RNA
vRNA	Viral RNA
RNP	Ribonucleoprotein
UTR	Untranslated region
ORF	Open reading frame
NEP	Nuclear export protein
RIDTs	Rapid influenza diagnostics
RT-PCR	Reverse Transcription polymerase chain reaction
FDA	Food and Drug administration
VTM	Viral transport medium
VL	Viral lysis
RNase	Ribonuclease
Mm	Milli molar
ddH2O	Double distilled water
TAE	Tris Acetate EDTA
EDTA	Ethylene diamine tetra acetate
UV	Ultraviolet

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Abstract

Influenza is an acute and potentially deadly respiratory virus that has an impact on public health globally and cause tremendous mortality and morbidity. In humans, recurring influenza epidemics are brought by two primary kinds of influenza viruses (A and B). Influenza has a unique seasonal pattern with clear peaks in the winter. Pakistan is one of those countries where influenza A is most prevalent and causes serious infections among all groups of ages. In this current study, the swab samples (n=25) were taken from patients having flu-like symptoms in Islamabad during the winter season of 2022-2023. The influenza samples were confirmed by PCR detection of the viral M gene. Subtype was confirmed by processing NA and HA genes and the H1N1 subtypes were identified throughout all samples. The identified N1 gene was further investigated through Sanger sequencing. Phylogenetic analysis of the current study sequences (isolate QAU-AS1) suggests clustering with a previously studied sequence from Washington and Nevada (Accession no. MK716148, MK399163); while isolate QAU-AS2 suggests clustering with sequence from South Africa (MN716554) representing an emerging subtype as compared to most of the reference sequences reported from the rest of the world. On the basis of current results, we suggest that the H1N1 subtype is still prevalent in Pakistan, and it needs to be investigated timely and effectively. The most productively initiative should be taken to speed up the vaccination process in the country. A further molecular study should be done for better understanding of HA as well as M gene of Influenza A virus.

CHAPTER 1
INTRODUCTION

1 Introduction

The world has suffered from influenza A pandemics, for a long period (Huremović, 2019). Even though prior epidemic records describe infections with clinical symptoms like influenza, it is ambitious to claim that these past epidemics were caused by evolving influenza viruses. current research has unveiled that these pandemic viruses emerge out from birds pool (Hannoun, 2013; Scholtissek, 1994). The developing influenza virus must be modified conditions before it can be transmitted to people. This adaptation might occur in a definitive host, such as poultry or swine (Russell and Webster, 2005). Pandemics appear to have begun very early in human history; some speculate that when human begun to use the fowls for food these viruses then become adapted to humans. First isolation and identification of influenza was done in 1933 by British scientists P.P. Laidlaw. C.H. Andrewes and Wilson Smith, at London's National Institute for Medical Research.

Influenza A virus belongs to the family of *Orthomyxoviridae* which is derived from the Greek word, *orthos* means correct, standard and *myxa* means mucus). *Orthomyxoviruses* are single-stranded, negative-sense RNA viruses having segmented genomes. The viral RNAs act as templates for the production of mRNA and cRNA. This family has been divided into four genera; influenza A, B, C, and D (Cheung and Poon, 2007; Krossøy et al., 1999).

The antigenic variations between the viruses of influenza A, B, and C nucleoproteins (Poovorawan et al., 2013) and matrix proteins (M) allow them to be characterized (Lamb, 2001). Influenza A and B have 8 segments of genome while C and D have 7 (Gao et al., 2012). All these viruses have potential to infect humans spontaneously. Till date only influenza A causes most of the pandemics (Nicholson, 1998; Nickol and Kindrachuk, 2019). Influenza Antigenic variation in the surface glycoproteins neuraminidase (NA) and hemagglutinin (HA) and further classifies influenza A viruses into subtypes. There are presently 16 recognized HA subtypes and 9 recognized NA subtypes (Laver et al., 1984; Tong et al., 2012). Surprisingly, these subtypes can all be isolated from seabirds, indicating that influenza viruses' native hosts are avian species (Ito and Kawaoka, 1998; Kim et al., 2018).

The HA contains five antibody binding domains or epitopes, denoted by the letters A through E, that are essential for antibody recognition (Plotkin et al., 2003). The HA has activity in the initial stages of viral infection, whereas the NA has activity in the latter stages of disease and the propagation of virions. NA is a 453 residue tetramer having enzyme sites

which cleave the terminal sialic regions of newly synthesized viral protein, allowing them to be released from host cells and preventing virus particle aggregation (Chauhan et al., 2022; Kaverin et al., 1998) as well as to facilitate viral release and dissemination (Stray et al., 2000). Hence, antibodies detect HA and NA, making them targets for antiviral medications.

The flu virus is also among the foremost causes of infection globally. During seasonal influenza epidemics, it is projected that 5-15% of the worldwide people becomes infected, resulting in one million fatalities per year (Organization, 2002; Yewdell et al., 2002). Pakistan is the sixth most populated country, with 40% of its people living in cities and little health care facilities, making it prone to influenza outbreaks. This malady is prevalent in Pakistan and badly affects people of all ages (Bukhsh et al., 2018). On June 18, 2009, the country's first H1N1 infection was recorded (Bashir Aamir et al., 2012). This new pandemic influenza A (H1N1) strain increased the influenza infection rate in 2009 to 27%, up from 21% in 2008. The influenza infection rate jumped from 18% to 28%, the highest in the country, between 2010 and 2011. Between 2008 and 2011, 1489 (22.8%) cases were positive in 6258 suspected samples, including 53% in Islamabad, 24% in Punjab, 13% in Sindh, 8% in Khyber Pakhtunkhwa and 2% in Baluchistan (Badar et al., 2013). Following the trend, the influenza virus took 444 lives over the year 2013 (Ahmed et al., 2019) 28 in 201,5 and 31 in 2016 (Farrukh et al., 2017).

Since 1980, the NIH (Islamabad) has conducted periodic influenza monitoring. A contract to advance and enhance influenza monitoring in Pakistan was signed in 2004 by the CDC and the NIH. This agreement calls for the surveillance of diseases like influenza and SARI by developing five geographically diverse surveillance sites and diagnostic facilities. Prior to CDC assistance, Pakistan had no sentinel lab based surveillance system (Ahmed et al., 2019).

Following the 2009 pandemic, the Pakistani government initiated effective influenza surveillance. Several directives have been issued by Pakistan's Ministry of Health (Atique et al., 2018). A crucial component of primary preventive measures is disease awareness among community and healthcare personnel. The Pakistani healthcare system is facing a huge issue in the form of inadequate health literacy, which is largely attributable to the country's low literacy rate (58% in 2017) (Yusufzai, 2018). There is no national data with up to date influenza vaccination numbers. The current state of influenza vaccine is deplorable, as evidenced by the grave scenario. In Pakistan, there is almost no national influenza vaccine

strategy, as evidenced by the failure of anti-influenza coverage initiatives to be planned and implemented (Ahmed et al., 2019).

Because the virus that causes the flu is continually evolving, the influenza vaccination is updated each year (Fonseca, 2019). In the U.S, the CDC and the WHO determine the influenza strains highly prone to spread during the upcoming flu season (Merced Morales et al., 2022) . Based on this data, the vaccination is designed to include the influenza viruses have ability to cause outbreaks during the forthcoming flu season. This implies that the vaccine should be upgraded every year to guarantee that it stays effective against the current influenza strains prevalent in the public (Morgon, 2022).

Our study aimed to inspect the influenza in individuals who have signs and symptoms like, sore throat, cough, fever, headache, muscles, or body-aches. Further, Polymerase chain reactions were performed to confirm the positive influenza samples and to explore the Neuraminidase (NA) gene of influenza isolates for nucleotides sequencing, its phylogenetic analysis, mutational analysis, and genetic assessment.

1.1 Aims and Objectives

1) To identify the circulating subtype of influenza virus in Islamabad.
2) To perform nucleotide sequencing and phylogenetic analysis of neuraminidase gene of influenza virus.

CHAPTER 2
REVIEW OF LITERATURE

2 Review of literature

2.1 Introduction to Influenza

The influenza virus causes an infectious illness that may affect either the upper or lower respiratory tracts in its victims. There are several different types of influenza viruses that might cause it. Some of these viruses are capable of infecting humans, while others are limited to infecting just certain animals. These viruses are transmitted by the respiratory droplets that are expelled from the mouth and respiratory system when an influenza patient talks, sneezes or cough. The influenza virus may be transferred by contacting contaminated objects and then touching one's own nose or eyes. It is possible to pass on the influenza virus both before and up to 5–7 days after signs of illness have appeared. Although the majority of healthy individuals will recover entirely within few days of being infected, certain high risk populations may have consequences like pneumonia and even death from the illness. These groups include infants and the elderly, those with impaired immune systems, and women who are pregnant. The common c old is characterized by a dry cough, runny or stuffy nose, high fever, and sore throat. During flu season, cases of the disease tend to spread rapidly and effectively. Epidemics of influenza occur in temperate nations every autumn and winter, affecting a significant number of children and adults, despite the fact that various seasons have varying effects on the age groups and severity of the disease (Auladell et al., 2019; Dos Santos, 2019; Marshall et al., 2019; Odun Ayo et al., 2018).

2.2 Etiology

A group of antisense RNA viruses are called *Orthomyxoviridae*, and influenza viruses belongs to that family (Paules and Subbarao, 2017). The influenza virus has a segmented genome (Paules and Fauci, 2019; Te Velthuis and Fodor, 2016). The influenza virus attaches to host cell's membrane and then breach it. The HA attaches to receptors present on cell surface, which opens the door for the virus to invade the cells (Byrd Leotis et al., 2017). Neuraminidase enzyme plays a vital role in replication of virus and assists in the process of the virus shedding and again its attachment to new host cell (Shtyrya et al., 2009). Therefore, pathogenesis and virulence are highly dependent on the glycoproteins.

Influenza consist of four distinct types of strains like: A, B, C, and D as shown in Table 1 (Bouvier and Palese, 2008). Most epidemics of influenza are brought on by only one or in

some cases two different strains of the influenza virus. The influenza A virus is a common kind of influenza virus that circulates throughout flu season. In humans and animals, influenza A may cause moderate to severe illness (Yoo et al., 2018; Yu et al., 2017). Influenza B virus has the potential to cause severe illness in certain people. There is several evidences that the influenza B virus is responsible for small but widespread outbreaks (Koutsakos et al., 2016). During the winter flu season, the incidence of influenza type B is much lower than that of influenza A (Chen et al., 2007). Humans are the most common hosts for type C viruses, however other species, such as pigs, may also get infected (Hause et al., 2013). Influenza C virus cause upper respiratory symptoms that are mild to moderate in severity, infrequent cases, and short localized outbreaks (Njouom et al., 2019). There is no evidence that viruses of type D may infect people; pigs and cattle are the primary hosts for these viruses (Foni et al., 2017).

Table 1: Epidemiology and targeted hosts of different subtypes of Influenza.

Types	Symptoms	Affect	Subtypes	Epidemiology
A	Benign to severe	Human and animal	9 NA and 16 H subtypes	Common worldwide
B	Benign	Only Human	Have no subtypes	Pandemics not reported
C	Benign	Human to some animals	Have no subtypes	Not even caused epidemics
D	Benign	Animals, unknown in humans	Have no subtypes	Not widespread

Type A viruses may be separated from other viruses based on the antigenic variation present in their NA and HA. There has been a total of 9 NA and 16 HA antigenic variants identified so far. In addition, many other permutations of HA and NA may be found in subtypes (like; H7N9, H5N1 and H1N1). Type B viruses have not been divided into subtypes (Paules and Subbarao, 2017).

2.3 Epidemiology

Flu may arise in several different ways, including outbreaks, epidemics, pandemics, and occasional cases. The epidemics often peak in the winter, both in southern and northern hemispheres (Tamerius et al., 2011). Pandemics of flu may strike at any time in a year in tropical locations (Paules et al., 2019; Sagripanti et al., 2007). Nearly half a million lives are lost annually due to influenza, and the WHO estimates that 4 million people get seriously sick due to annual outbreaks (Clayville, 2011). Virus's ability to propagate, new strain and population's susceptibility to that new strain, all play major part in the epidemiologic pattern of infection, which in turn reflects the virus's mutating antigenic features. Antigenic shifts occur when extensive and quick changes are made to surface glycoproteins of some animal strains after they have been genetically transferred to human strains (Shao et al., 2017). Even though antigenic shift is very unusual, it has the potential to spark widespread disease outbreaks. Mild antigenic changes within the HA or NA of influenza are common and are commonly associated with regional outbreaks (Stellrecht, 2018). In Influenza B infections, antigenic drifts only within the HA have been detected. Anyone may get influenza, but the old aged are at a higher risk for severe complications, hospitalization, and death (Taubenberge et al., 2008). Complications from influenza during pregnancy, especially pneumonia, have been associated to increased maternal mortality and disability (Mertz et al., 2017).

Please keep in mind that birds are significant reservoir of influenza virus. Human cases of avian flu caused by subtypes H9, H7, and H5 have all been documented. H5N1, caused enormous fatality in humans with an estimated rate of mortality about 50% among these strains (Neumann, 2015). As many as 36 people lost their lives in 2013 when there was an outbreak of H7N9, even though it is mostly harmless to birds. So, because H7N9 pandemic in humans is possible due to the virus' rapid transmission, it's crucial to treat such illnesses as serious threats to public health (Poovorawan et al., 2013; Sivanandy et al., 2019).

2.4 Structure of influenza virus

The morphology of influenza means the shape, hereditary features, and its various proteins (NA, HA, M1, and M2). So the shape of the influenza virus range from spherical to long filamentous in size as shown in Figure 1 (Burleigh et al., 2005; Roberts et al., 1998).

The surrounding lipid envelope is produced when the virus buds out from the host's cell. This surrounding lipids coat contains three epitopes, NA, HA, and M2 (Lamb, 2001). NA and HA are glycoproteins present on that lipid bilayer and attached by hydrophobic amino acid. HA is a homotrimer that has the ability of membrane fusion and receptor binding. While NA is a homotetramer with the role of destroying receptors and releasing viral progeny by hydrolyzing sialic acid (Hutchinson, 2018).The M2 protein acts as pathway for ions through which acidification process of viral particle's interior become possible (Chauhan et al., 2022; Pinto et al., 1992; Wang et al., 1994). Beneath the lipid bilayer there is M1 protein layer (Hui et al., 2022)

Inside the virion, all segments of vRNA are attached to the RNA polymerases and Nucleoprotein to form RNP complexes (Lamb et al., 1983; Lo et al., 2018). Aside from M1, NP protein is mostly present in the Influenza virus, which interact with the sugar phosphate backbone of the viral RNA in a sequence independent way (Baudin et al., 1994). Every NP monomer have connection with about 20 vRNA nucleotides (Lamb, 2001). The Three polymerase subunits combine and make the RNA polymerase complex (PA, PB1, and PB2). Electron micrographs revealed that a supercoil shape is produced by vRNA through end to end interaction (Cheng et al., 2017; Hsu et al., 1987), one end of RNP contains RNA polymerase, suggesting RNA polymerase appears to interact with both edges of vRNA inside the viruse. Virions contain NS2 in trace concentration (Richardson et al., 1991).

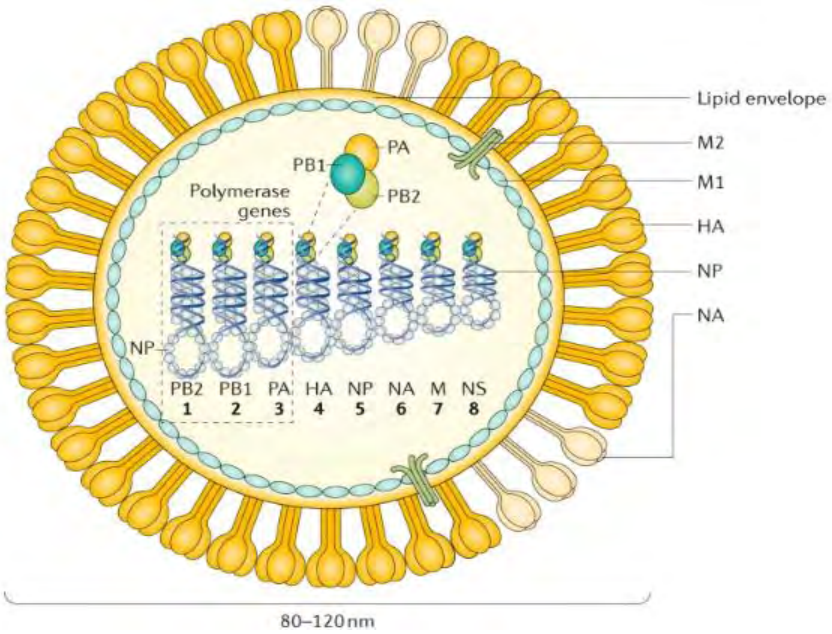


Figure 1: Morphological structural of Influenza A and B virus (Krammer et al., 2018).

2.5 Genomic Organization of Influenza.

As shown in Figure 2, eight gene segments are present in both A and B Influenza (McGeoch et al., 1976) they provide mRNAs for ten necessary viral proteins and large number of strain specific structural proteins (Lamb, 2001).

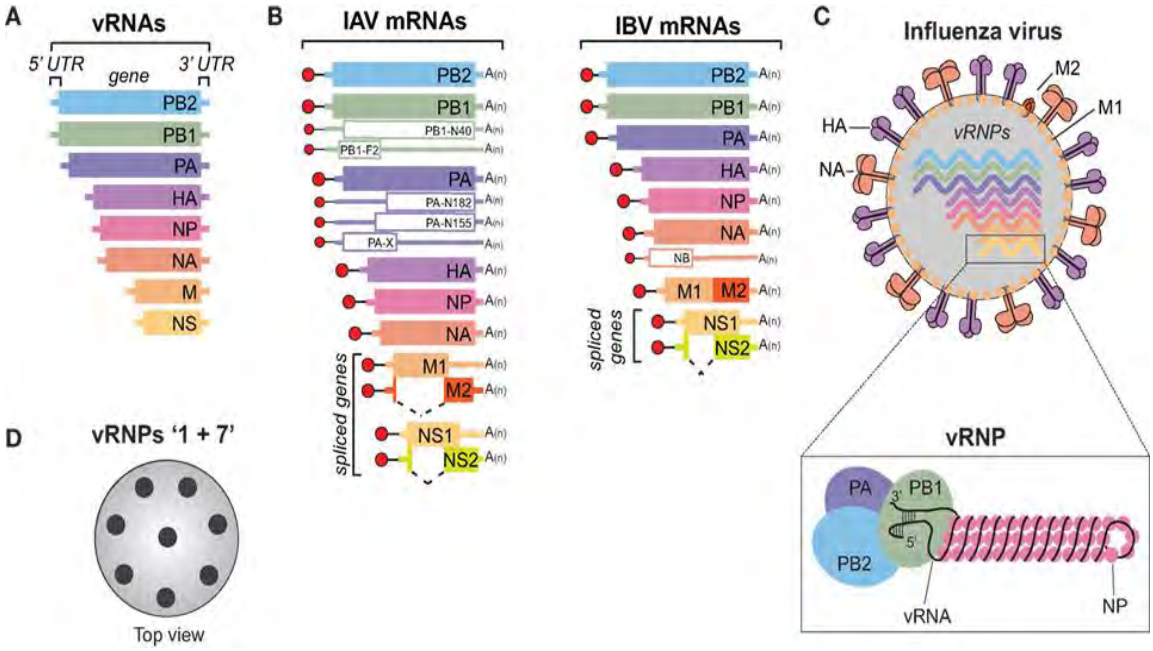


Figure 2: Genomic representation of Influenza A, B and C Virus. (A) Representing eight segments that make up the influenza genomes are shown in a diagram. Lines are used to depict the viral promoter containing 5′ and 3′ UTR, and boxes are used to indicate the each vRNA coding area. (B) mRNAs that are produced from the IBV (right) and IAV (left) templates of vRNA. The alternate splicing of the IAV NS and M transcripts, in addition to the IBV NS transcript, is shown by the dashed lines and boxes, which represent the viral gene product that each mRNA encodes 10–13 nucleotide, primers that are derived from the host acquired by the viral polymerase's cap-snatching process are indicated by black lines and red circles, which show the 5′ M7pppG cap (McGeoch et al., 1976).

Segments 1 and 3-6 of viruses include monocistronic mRNAs. Segment 2 mRNAs from some viral strains have been shown to have a second ORF (open reading frame). However, viral mRNAs synthesized from segments 8 or 7 are capable of undergoing alternative splicing, leading to the synthesis of viral proteins. Proteins and their segments are summarized in Table 2. The only non-structural proteins are NS1 and PB1 F2 from segment 8 and segment 2 respectively (Lamb, 2001).

Table 2: The influenza virus's RNA and the correspondingly translated proteins.

Segments of viral RNA	Length of viral RNA (bp)	Protein(s) encoded
Segment-1	2341	PB2
Segment-2	2341	PB1 & PB1-F2
Segment-3	2233	PA
Segment-4	1778	HA
Segment-5	1565	NP
Segment-6	1413	NA
Segment-7	1027	M1 & M2
Segment-8	890	NS1 & NS2 (NEP)

2.5.1 Basic Polymerase Protein 2 (PB2) Segment 1

A influenza's first segment encodes PB2, a viral polymerase component (Honda et al., 2002; Perales and Ortín, 1997). By virtue of its nuclear localization signal, PB2 is translocated into the nuclei of cell which has been infected, where it participates in viral replication and transcription (Jones et al., 1986; To et al., 2019).

2.5.2 Basic Polymerase 1 (PB1) Segment 2

In segment 2, you may find the code for the PB1 subunit of RNA polymerase. PB1's potential role as an RNA polymerase was supported by many lines of evidence. PB1 transports the location for RNA polymerase (Poch et al., 1989). Several studies revealed the specific regions of PB1 that interact with other polymerase subunits. Studies using the RNA polymerase's immunoprecipitation method have revealed that PB1 has unique binding regions for PA and PB2 (Cheung et al., 2020; Digard et al., 1989).

2.5.3 Acidic Polymerase (PA) Segment 3

Segment 3 encodes the smallest component of RNA polymerase complex called PA proteins. In all the influenza viruses this subunit of polymerase has nuclear localization signals essential for entering to the nucleus (Jones et al., 1986; Lutz Iv et al., 2020). Several studies have shown that PA is necessary for viral replication and transcription. A polymerase

complex containing PA, PB2, and PB1 has been revealed to be necessary for viral replication and transcription in several studies (Bu et al., 2021; Huang et al., 1990)

2.5.4 Hemagglutinin (HA) Segment 4

To continue with the flu, we now have the fourth segment which encodes hemagglutinin. The HA proteins often fold into homotrimer. This protein mediates viral particle attachment host cell receptors. In addition, it also have a role in the process through which viral and cellular membranes fuse (Lamb, 2001; Nicholson, 1998). Additionally, it acts as the main site for antibodies that fight infections (Sherry et al., 2016; Staudt and Gerhard, 1983).

2.5.5 Nucleoprotein Segment 5

NP is encoded in segment 5. The protein has overall positive charge and is phosphorylated at neutral pH (Huang et al., 1990; Perales and Ortín, 1997). The amino terminal of the NP protein contains RNA binding region. It also control the activity of RNA polymerase which become shifted from transcription to replication (Shapiro and Krug, 1988).

2.5.6 Neuraminidase (NA) Segment 6

The Influenza genome's 6th segment encodes the NA. As of right now, the exact function of neuraminidase in the influenza is unknown (Crescenzo Chaigne et al., 2017; Liu et al., 1995). It is now generally accepted that NA performs a crucial function in assisting the dispersal of virus progeny from the infected host cells. Current evidence reveals that NA's conserved cytoplasmic tail controls virion shape and pathogenicity (Bilsel et al., 1993; Diefenbacher et al., 2022; Jin et al., 1997).

2.5.7 Matrix Proteins (M1 and M2) Segment 7

Segment 7th of Influenza Both the M1 and M2 proteins are virally encoded. As a collinear transcription product, the M1 protein may be traced back to segment 7. Conversely, segment 7 mRNA splicing results in the production of M2 protein (Kordyukova et al., 2019; Shih et al., 1995).

2.5.8 M1 Protein

This protein plays a major role inside the virus to separate the ribonucleoproteins from membrane of the virus by creating a layer. This M1 protein interacts also with vRNA as well as RNP (Ruigrok et al., 2000).

2.5.9 M2 Protein

M2 is found as a disulphide-bonded homotetramer and is an embedded membrane protein (Manzoor et al., 2017; Sakaguchi et al., 1997). The M2 tetramer has pH regulating ion channel activity. Transmembrane domain of the M2 protein contains the amino acid residues Tryptophan (Trp41) and Histidine (His37), which are essential for conducting of protons. M2's ion channel activity in infected cell endosomes acidifies the incoming viral particle (Takeuchi et al., 2003). Acidification of the viral particle is necessary for replication because it enables arriving vRNPs to disengage from M1 proteins for nuclear importation (Bao et al., 2022; Bui et al., 1996; Martin and Heleniust, 1991).

2.5.10 Non-structural Proteins (NS1 and NS2) Segment 8

This segment is responsible for encoding NS1 and NS2 proteins. In this case, NS2 is spliced version of mRNA while NS1 is a collinear transcriptional product (Alonso Caplen and Krug, 1991; Hao et al., 2020; Nemeroff et al., 1992).

2.5.11 NS1 Protein

NS1 is a sole nonstructural protein which influenza have. It mostly exists as a nuclear oligomer. By binding to various RNA molecules, NS1 protein seems to control the production of both viral and cellular proteins (Han et al., 2019).

2.5.12 NS2 Protein

As its name implies, NS2 is a protein that was at first assumed to be nonstructural. However, other studies have shown that NS2 is present in virus particles only at low concentrations (Lin et al., 2022; Richardson and Akkina, 1991; Yasuda et al., 1993). Based on several studies of NS2 mutants, (Odagiri et al., 1994) suggest that NS2 promotes accurate replication of genomic RNAs, however how it does so is unclear.

2.6 Transmission

Typically, the flu virus is passed to another person by respiratory droplets such as coughing and sneezing (Cowling et al., 2013).

There are three ways to get a virus:

(1) Proximity to an infected person.

(2) Handling an infected item.

(3) Breathing in contaminated air.

Virus particles in the air may be inhaled and inhaled by others by any number of activities, including coughing, sneezing, talking, singing, and even just breathing (Cowling et al., 2013; Killingley et al., 2016). Human lower respiratory tract viral multiplication is correlated with the intensity of infection as it is associated with significant inflammation brought on by immune cells' infiltration (Fouchier et al., 2004).

Preventing the spread of viruses requires taking precautions such as covering mouth and nose while coughing or sneezing and washing hands thoroughly with water and soap or alcohol containing cleaner (Killingley and Nguyen Van Tam, 2013).

2.7 Cell entry and viral replication

2.7.1 Virus attachment

H1N1 influenza viruses can infect humans because they recognize N acetylneuraminic acid on the surface of the host cell. Glycoconjugates often have sialic acids, which are acidic monosaccharaides with nine carbons, attached to their terminals. Therefore, they are present on many kinds of cells and in many kinds of animals. Different steric topologies of the terminal sialic acid emerge from the carbon-2 forming -2,6- or -2,3-linkages with either the carbon-6 or carbon-3 of galactose. The influenza virus surface HA spikes preferentially identify and bind -2,3- or -2,6-linkages, which contain the sialic acid moiety. Epithelial cells lining the human trachea are mostly characterized by -2,6-linkages, whereas those lining the duck gut are predominantly characterized by -2,3-linkages. Human respiratory epithelium contains sialic acids with terminal -2,3-linkages, but at lower concentrations than those with terminal -2,6-linkages (Couceiro et al., 1993; Matrosovich et

al., 2004a); This concluded that avian influenza viruses may infect humans and other primates, though with reduced efficiency compared to human strains (Beare and Webster, 1991; Dou et al., 2018; Murphy et al., 1982; Tian et al., 1985).

Some avian strains have low infectivity but high pathogenicity, and this may be because of varied production of sialic acids in respiratory system of mammals. Lower respiratory tract -2,3-linked salicylate proteins are more common than in the rest of the human body (bronchioles and alveoli). As opposed to the upper respiratory system (trachea, bronchi, nasopharynx, and Para nasal sinuses), the lungs are a less likely site of infection for people exposed to avian viruses. Death rates in clinical settings with avian strains reach 60% because to the severity and rapid progression of the pneumonia they cause (Gambotto et al., 2008).

The hemagglutinin molecule is a trimmer composed of two different regions: triple stranded stem and globular head (Wilson et al., 1981; Wu et al., 2020). Head region contains the sialic acid receptor binding site, and it also have variable regions Cb, Ca1, Ca2, Sa, and Sb that could act as epitopes in upcoming new endemics as shown in Figure 3 (Fields, 2007). Because anti HA antibodies neutralise viral infection, amino acid alterations at antigenic regions are common across virus strains. Despite this, the structure of HA molecule is conserved among different subtypes and strain. When mutations in several antigenic sites occurs simply says that when antigenic drift occurs, then the host become susceptible to that new virus because his antibodies no longer recognizes that strain. During viral replication, serine proteases cleave the HA protein in half, creating HA2 and HA1. This particular post translational modification is essential for virus infectivity. In contrast to the HA1 segment, which contains antigenic and receptor binding (Steinhauer, 1999; Tsai et al., 2019).

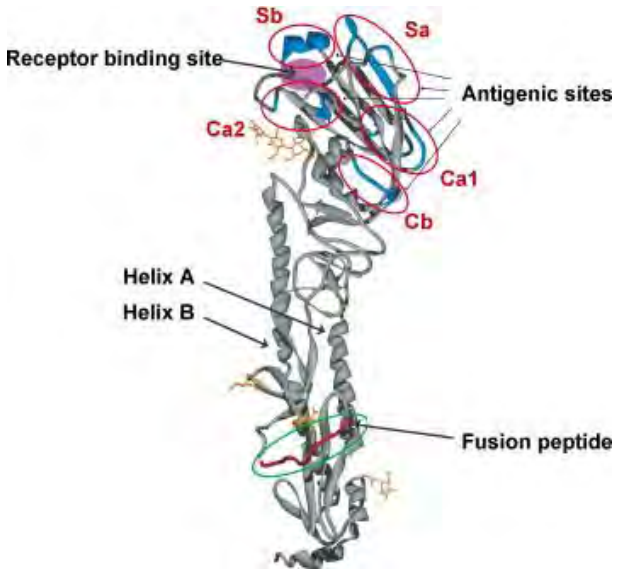


Figure 3: Sialic acid located the head, which contain five anticipated antigenic sites (Cb, Ca2, Ca1, Sb and Sa). As indicated, the stem is made up of helices A and B as well as fusion peptide. (Fields, 2007).

2.7.2 Entry of virus

The flu virus is engulfed once the HA protein binds to sialic acid as show in Figure 4. In two different ways, the acidity of endosomal section is crucial for the genome. When the cytoplasmic pH of host cell is low a geometrical change occurs in HA. As a result fusion peptide exposed to attaches the e host cell, viral RNPs then entered to the host cell cytoplasm (Sieczkarski and Whittaker, 2004; Stegmann, 2000). A class of anti-influenza drugs called amantadine targets the M2 protein. (Luo, 2012; Pinto et al., 1992; Wharton et al., 1994); and it has also been suggested as a component of vaccines due to its status as a surface protein (Neirynck et al., 1999) The M2 channel is responsible for disassembly of influenza internal protein-protein interactions, releasing viral RNPs from the viral matrix and into the cellular cytoplasm (Martin and Helenius, 1991; Skehel et al., 2000).

2.7.3 Viral RNA synthesis

RNPs and also other viral proteins are directed to enter the host cell nucleus after being released from the virion by nuclear localization signals on viral proteins (Cros and Palese, 2003). Host cell translational machinery uses the genome segments along with capped polyadenylated mRNA for synthesis of viral progeny. A component of the RNPs and RdRps makes two sense strands using antisense vRNA.

The polymerase enzyme of influenza converts the poly A tail present in vRNA into amino acid string of adenosines. This process contrasts with the polyadenylation of mRNA of host cell, which is carried out by poly(A) polymerase (Li and Palese, 1994; Luo et al., 1991; Robertson et al., 1981; Skehel et al., 2000). To start the production of viral mRNA, the PB2 and PB1 proteins take 5′ capped primers from host pre mRNA transcripts, a process analogous to messenger RNA capping (Krug, 1981; Weis et al., 2021).

Viral mRNA, once polyadenylated and capped, undergoes the same export and translation processes as host messenger RNA. But viral proteins NS2/NEP and M1 control nuclear export of viral RNA segments. M1 connects with the NEP, This permits M1 RNP into the cytoplasm, and M1 interacts with both NP and vRNA, leading to the idea that Within the RNP complex, M1 combines these two elements (Cros and Palese, 2003).

2.7.4 Synthesis of viral proteins

From viral mRNA, membrane bound ribosomes translate and export the surface proteins NA, HA and M2, which are then carried out to the endoplasmic reticulum for folding and trafficking to the Golgi apparatus for post translational modification. The three proteins are guided to the cell membrane by distinct apical sorting signals, where they help form the virion. Prior to the packaging at the host cell membrane, M1 is assumed to play a contribution in bringing the RNP NEP complex into interaction with the surface bound NA, HA, and M2 proteins (Fields, 2007).

2.7.5 RNA packaging and viral assembly

According to recent research, vRNA packaging is a more selective mechanism that ensures a complete genome, whereas previous research suggested that the packaging was completely random, with genomic RNA segments being incorporated into budding virus particles at random and only those having full genome becoming infectious (Bancroft and Parslow, 2002; Duhaut and Dimmock, 2002; Enami et al., 1991).

2.7.6 Virus budding and release

The cytoplasmic side of the lipid bilayer is where influenza virus budding first occurs, most likely as a result of a buildup of M1 matrix protein. After the virus has budded, the HA spikes firmly attach it to the sialic acid on the surface of cell, where it stays until the sialidase activity of the NA protein actively discharges the viral particles. The NA is a tetramer with a

mushroom shape and a transmembrane domain that binds to the viral envelope (Colman et al., 1983; Dawson et al., 2018; Varghese et al., 1983). It degrades receptors by cleaving terminal sialic acid residues from surface of cell gangliosides and glycoproteins in order to release viral progeny from the host cell. When neuraminidase inhibitors or viruses with missing or inactive NA are present, virus particles clump together at the cell surface, reducing their ability to infect cells. Further, the NA removes sialic acid residues from the virus membrane, which stops viral particle aggregation and boosts infectivity (Hutchinson, 2018; Palese and Compans, 1976; Palese et al., 1974). It has also been postulated that the NA aids viral infectivity by degrading mucins in respiratory tract secretions and therefore facilitating viral entry into respiratory epithelial cells. Neutralizing NA antibodies produced by the host and neuraminidase inhibitors prevent virus release from infected cells and, in turn, reduce viral multiplication (Matrosovich et al., 2004b).

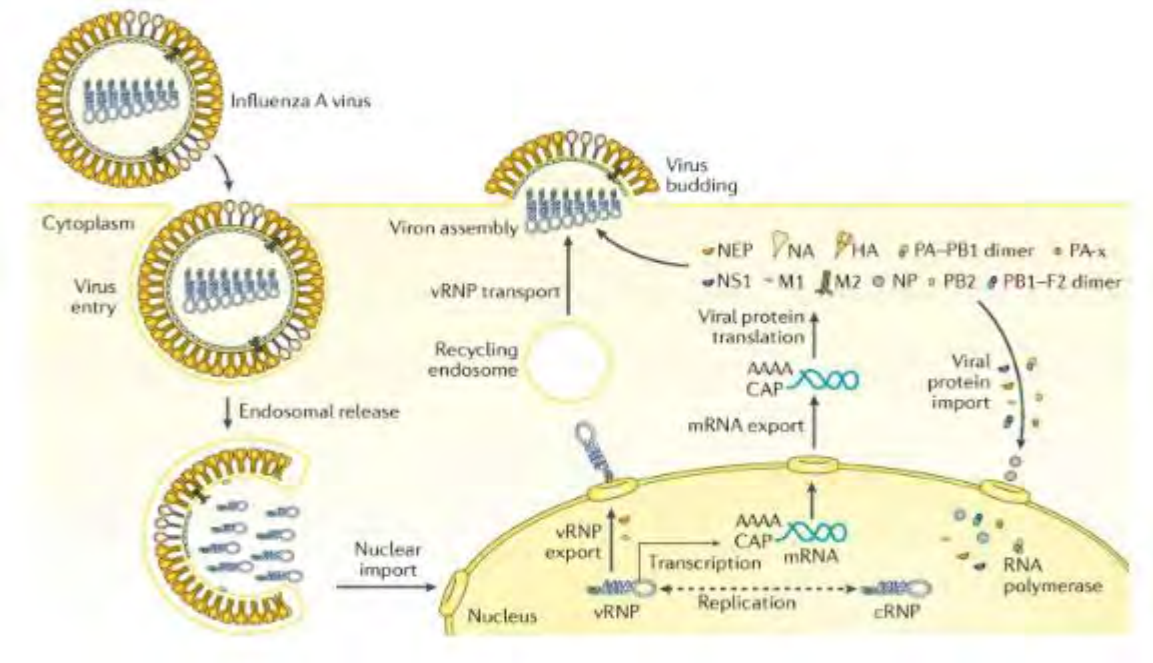


Figure 4: Influenza Virus life cycle (Krammer et al., 2018).

2.8 Flu virus "Drift" and "Shift" mechanisms

The influenza virus is continually evolving. They can transform in two ways.

2.8.1 Antigenic Drift

Antigenic drift describes the mechanism through which influenza viruses change. Subtle shifts (or mutations) in the influenza viral genome may produce drift, which in turn

can modify the NA and HA proteins on the virus's surface (Figure 5). The NA and HA surface proteins of flu viruses are both recognized by the immune system as antigens and may elicit an immune response, including the production of antibodies that can be used to fight infection. As influenza viruses replicate, they constantly undergo changes due to antigenic drift. Flu viruses that have undergone just a few mutations tend to cluster together on phylogenetic trees because of their tight genetic relationship. Similar influenza viruses have similar antigenic features. This means that the antibodies your body creates in response to one strain of influenza will likely recognize and react to other strains of influenza that share the same antigen (a process known as "cross-protection").

Small changes in NA and HA may add up over time to produce viruses that are antigenically distinct, which means that a person's antibodies attach to the virus in a different way or not at all, resulting in a loss of protection against that flu strain. With just one little alteration, the HA of a flu virus can change antigenically. Whenever a flu virus becomes antigenically different, or "antigenically drifts," it usually indicates that perhaps the virus's antigenic characteristics are considerably distinct and that the body's immune system (specifically, an individual's existing antibodies) will have a tougher time identifying and tackling the virus (CDC report, 12/Dec/2022).

2.8.2 Antigenic Shift

The term "antigenic shift" describes yet another kind of mutation. A shift is a rapid, noticeable change during epidemics. An infectious agent that modifies the influenza virus genome to create novel HA and/or NA proteins. One possible outcome of an antigenic shift is a novel influenza strain. There is a new strain that has infected a human. If a flu virus formerly seen in animals develops the ability to infect humans, this would be a significant change. There is a possibility that viruses originating in animals may contain HA/NA or HA combinations that are so different from those of human viruses that most people won't be immune to the novel virus (CDC report, 12/Dec/2022).

2.10 Complicacies with the flu

Most people who have the flu get well in a week or less, but a small percentage develop serious consequences (like pneumonia) that may be deadly.

Minor flu complications include ear infections and sinus, but pneumonia is a severe flu complication that may be brought on by a flu virus infection alone or by a flu virus and bacterial co-infection. Aside from multi organ failure, the flu may cause inflammation of the brain (encephalitis), heart (myocarditis), or muscles (rhabdomyolysis, myositis) (for example, kidney failure, and respiratory diseases). Sepsis, the body's potentially lethal response to infection, may be triggered by a respiratory tract infection with the influenza virus. The flu has the potential to make pre-existing medical issues worse. The flu may exacerbate pre-existing conditions in some individuals, such as asthma, and cause symptoms in others (CDC report, 18-Nov-2021).

Table 3: Signs and symptoms of flu and cold.

Signs & Symptoms	Influenza	Cold
Symptoms onset	Abrupt	Gradual
Sneezing	Common	Common
Nasal congestion	Common	Common
Pharyngitis	Common	Common
Tiredness, weakness	Sometimes	Sometimes
Chest pain, cough	Mild to ordinary; hacking cough	Mild to ordinary; hacking cough
Chills	Uncommon	Uncommon
Headache	Rare	Rare

2.11 Diagnosis

The detection of flu viruses in respiratory samples may be done using a variety of assays. The most widely used are "rapid influenza diagnostic tests" (RIDTs). RIDTs do their job by picking up on antigens (viral parts) that set off an immune response. These tests may not be as accurate as more time consuming lab-based methods, but they may provide results

in as little as 10 to 15 minutes. Therefore, you may still have the flu even if a rapid test comes out negative. According to a group of researchers (Morehouse et al., 2022). "Rapid molecular assays" are another kind of flu test that can detect the virus's genetic material. Rapid molecular tests, which provide answers in 15–20 minutes, are more precise than RIDTs (Munoz, 2022).

More precise influenza testing, such as RT-PCR and rapid molecular assays, are performed only in specialized facilities like those found in hospitals and health departments. One may be tested using a variety of methods, such as a reverse transcription polymerase-chain reaction (RT-PCR), a viral culture, or an immunofluorescence assay. A swab is used to clean the nasal passages or the back of the throat by a medical practitioner, and then the sample is sent off to the lab for analysis. The time it takes to get a response might be anything from a few to a few dozen (CDC report, 18-Nov-2021).

2.12 Treatment

Prescription antiviral drugs are available for treatment of influenza. Those with pre-existing conditions like as asthma, diabetes (including gestational diabetes), or heart disease should get treatment immediately possible to reduce their risk of serious flu complications (CDC report, 14-Dec-2022) .

This flu season, the CDC recommends using four antiviral medications that have been authorised by the FDA.

- Baloxavir marboxil (trade name Xofluza)
- Peramivir (trade name Rapivab)
- Oseltamivir phosphate (Tamiflu)
- Zanamivir (trade name Relenza)

2.13 Influenza Vaccines

There is no substitute for the flu shot, and antiviral drugs are not it. The most effective defence against seasonal flu and its possibly catastrophic consequences is to be vaccinated against it. Every person aged 6 months and above should receive a flu vaccination every year. Antiviral drugs are a secondary line of protection against the flu (both seasonal flu and flu mutant viruses) (Control and Prevention, 2018).

2.13.1 Vaccine development for influenza

In 1931, Ernest William Goodpasture and his colleagues at Vanderbilt University observed viral replication in fertilized hen eggs. Wilson Smith, Jonas Salk, Macfarlane Burnet and Thomas Francis, are among the researchers who expanded their focus to encompass the evolution of influenza viruses; their work led to the creation of the first influenza vaccines in the laboratory (Plotkin et al., 2004). It was during World War II that the U.S. military produced the first approved inactivated influenza vaccinations (Artenstein, 2010). Manufacturers still used hen's eggs as a source of influenza virus, but they developed more effective methods to remove egg proteins and reduce the vaccine's systemic reactivity, leading to a purer virus and a safer vaccine (Hampson, 2008). In 2012, the FDA approved cell culture derived influenza vaccinations for use in the United States (Milián and Kamen, 2015), and recombinant proteins influenza vaccines (Food and Administration, 2013) Clinical trials of plant based influenza vaccinations are now underway, (Landry et al., 2010).

CHAPTER 3
MATERIAL AND METHODS

3 Material and methods

3.1 Samples collection

With informed permission, nasopharyngeal/nasal and throat samples/swabs were taken. Swabs were sterile Nylon, rayon, or dacron with plastic shafts that were carried in 2-3 ml of viral transport medium (VTM) and kept at -70 °C before processing, and samples were shipped to the Infectious Diseases and Molecular Pathology lab of Quaid-i-Azam University for laboratory analysis.

3.2 Criteria of inclusion

Samples were collected from all those patients who have flu-like symptoms.

3.3 Criteria of exclusion

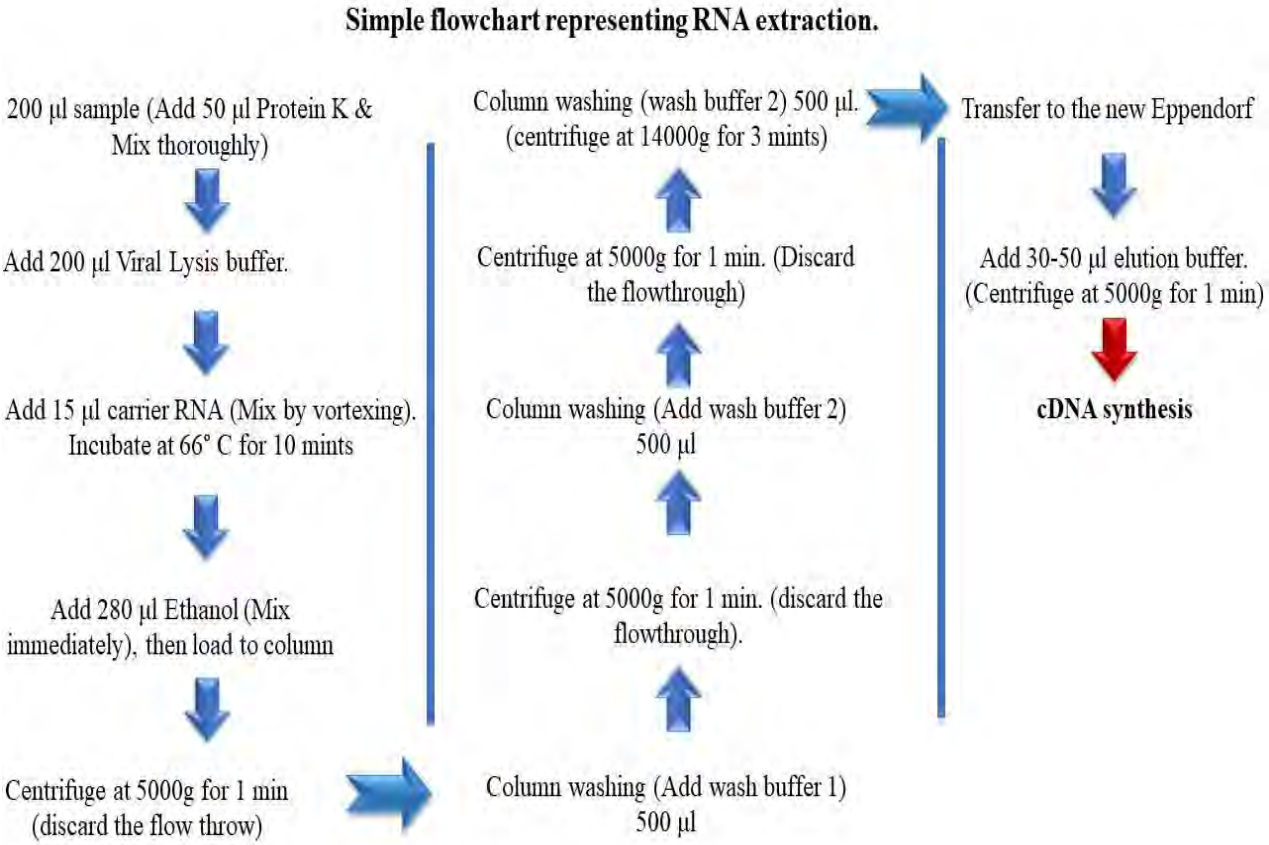
Those patients who were negative for Influenza virus were excluded.

3.4 RNA Extraction

Viral DNA/RNA extraction mini kit (WizPrep) was used for the extraction of influenza RNA, following the standard manufacturer's protocol. The nasopharyngeal swab was transferred to an Eppendorf tube of 1.5ml and centrifuged for 1 minute at 8000 rpm. After centrifugation, two layers were formed, then the lower layer was transferred into a new autoclaved microfuge tube.

First, sterile microfuge tubes were labelled using specific lab codes. The labelled microfuge tubes of 200µl serum samples were added after that sample's lysis started by the addition of 50µl proteinase k and then the sample was mixed thoroughly. From the viral RNA/DNA extraction mini add 200 µl of VL buffer and then 15µl of carrier RNA were added into the tube containing the sample and mixed for 10-15 sec by vertexing. The sample mixture was incubated for 10 mins at 66°C in the pre heated block, after the incubation step, 280µl of the absolute ethanol was added into each tube and mixed immediately to prevent precipitation of ethanol due to its concentration. After that, the sample mixture was transferred into the column provided in the kit and was centrifuged at 5000g for 1 min, after the centrifugation the flowthrough was discarded, and washed the column with 500µl of wash

buffer 1, and was centrifuged again for 1 min, at 5000g, and the flowthrough was discarded. The column-was washed again with 500µl wash buffer 2 provided in the kit, centrifuged at 5000g for 1 min, and discarded the flowthrough. Again, the column was washed with 500µl wash buffer and centrifuged at 14000g for 3 mins, the flowthrough was discarded. The column was transferred into a new microfuge tube, 30-50µl elution buffer was added, and centrifuged for 1 min at 5000g, the viral RNA was stored at -20°C.



3.5 Influenza complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesized using the extracted RNA as a template. First strand cDNA Synthesis Kit (RevertAid, ThermoScientific) was used. The components for cDNA synthesis were as follows: 8µl of extracted RNA along with 2µl Random hexamer or Uni-12 and Uni-13 primers (Tseng et al., 2014; Yan et al., 2022), 2µl double distilled water, 2µl dNTP (10 mM), 4µl 5x reaction buffer, 1µl RevertAid RT enzyme (200 U/µL) and 1µl RiboLock (20U/µL) RNase Inhibitor were mixed gently, and total reaction volume

adjusted to 20 µl as shown in Table 1. All the components of reaction mixture were mixed and incubated for 5min on 25°C, followed by 42°C for 60 min, 45°C for 30 minutes and finally reaction was completed at 70°C for 5 mins as shown in Figure 6.

Table 4: Conditions optimized for Influenza complementary DNA (cDNA) synthesis.

Serial No.	Reagents	Quantity
1	5x reaction buffer	4µl
2	Uni 12 or Uni 13 primers	2µl
3	10 mM dNTP Mix	2µl
4	Template RNA	8µl
5	ddH2O	1µl
6	RiboLock (20U/µL)	2µl
7	RevertAid RT enzyme (200 U/µL)	1µl
8	Total volume	20µl

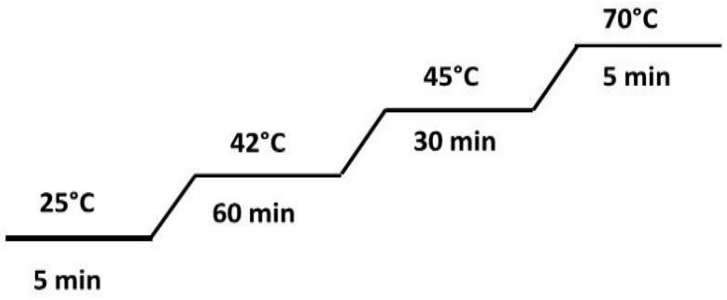


Figure 6: Representation of optimized conditions for cDNA synthesis.

3.6 PCR based detection through M gene of influenza

Nested polymerase chain reaction of M gene of influenza was carried out using M-229L as forward and M-229U as reverse primer suggested by Bi and coworkers (Bi et al., 2016) to identify whether the samples were influenza positive or negative. 20 µl reaction mixture was made using 2x Phusion HF master mix. The components and their quantities used in the PCR mixture are given in below Table 2. The cyclic condition for PCR were as follows: 95°C for 5mins following 35 cycles of 95°C for 45 seconds, 57°C for 30 seconds,

70°C for 1 mint and final extension at 70°C for 10 min as shown in Figure 7. The reaction was hold at 4°C for infinity.

Table 5: Reaction mixture of M gene's PCR.

Components	Volume
Phusion Master Mix	10 µl
Forward Primer	1.5 µl
Reverse Primer	1.5 µl
dH2O	1 µl
Template	6 µl
Total	20 µl

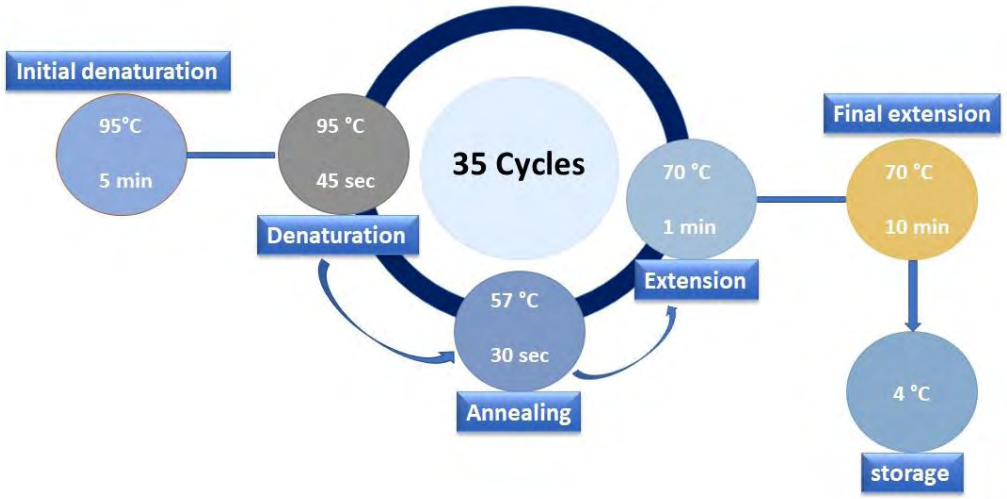


Figure 7: Polymerase chain reaction's cyclic conditions for M gene partial region.

3.7 PCR amplification of Influenza N gene

Several polymerase chain reactions were carried out to identify the subtype of N gene of influenza, as the influenza virus has been classified into 11 NA subtypes (N1-N11). That's why 5 sets of different primers were applied on every sample and annealing temperature of every primer were given according to the previous studies. In all these samples N1 primer

gave positive results. PCR protocol of N1 gene is given below. The cyclic condition for PCR of N gene were as follows: 95°C for 5mins following 35 cycles of 95°C for 45 seconds, 53°C for 30 seconds, 70°C for 1 mint and final extension at 70°C for 10 min as shown in Figure 8.

Table 6: Reaction mixture of N gene's PCR

Components	Volume
Phusion Master Mix	10 µl
Forward Primer	1.5 µl
Reverse Primer	1.5 µl
dH2O	1 µl
Template	6 µl
Total	20 µl

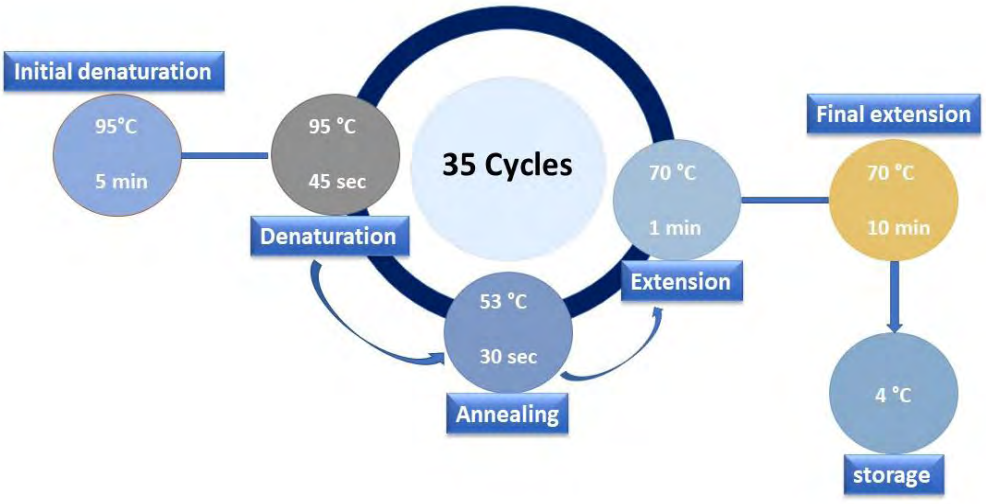


Figure 8: Polymerase chain reaction's cyclic conditions for N gene partial region.

3.8 PCR amplification of Influenza H gene

Several polymerase chain reactions were carried out to identify the subtype of H gene of influenza, as the influenza virus has been classified into 18 HA subtypes (H1-H18). That's why 4 sets of different primers were applied on every sample. In all these samples H1 primer

gave positive results. PCR cyclic conditions were optimized using Gradient PCR. Annealing temperature was optimized at 56°C for 30 seconds as shown in Figure 9.

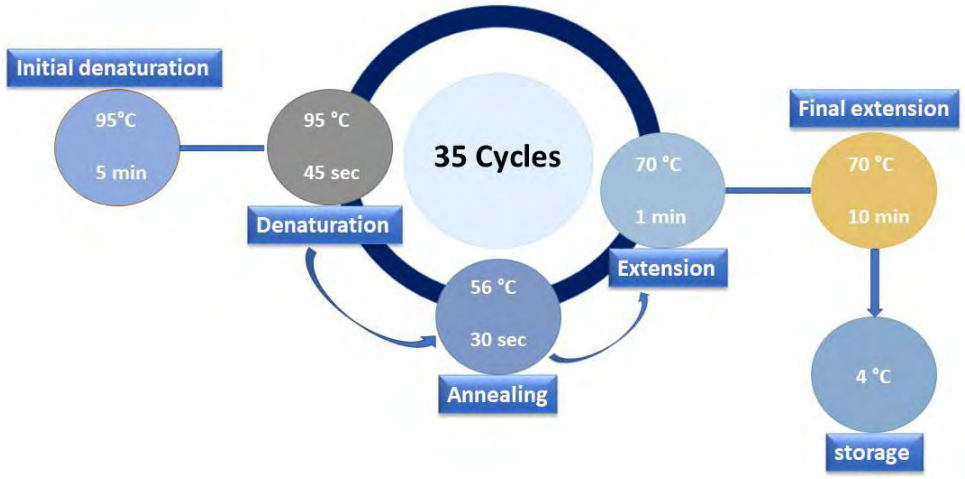


Figure 9: Polymerase chain reaction's cyclic conditions for H gene partial region.

3.9 Preparation of TAE buffer

TAE is a short form of Tris Acetate EDTA (Ethylenediaminetetraacetic acid), a running buffer which is employed to separate DNA in agarose gel during the process of gel electrophoresis. Reagent like Tris base, glacial acetic acid, EDTA and double distilled water (ddH2O) are used in preparation of TAE buffer. For the preparation of 10X TAE buffer in 1 litter 48.4g of Tris base and 11.4ml of glacial acetic acid was added to 800ml of ddh2O and mixed thoroughly. Then 3.7g of EDTA was added to 200ml of ddH2O for the confirmation of pH of EDTA. When EDTA completely dissolved in water and become transparent, this transparency means that pH of EDTA is normal. Both of the mixtures were mixed then, to make it 1000ml final solution. To make 1X TAE buffer (working buffer), 10ml of the prepared 10X buffer was added to 90ml of ddH2O to make 100ml of 1X TAE buffer.

3.10 Gel electrophoresis for confirmation of M gene

For confirmation of amplified PCR products, they were run on 1.5% agarose gel. The gel was prepared in a 60ml (1X TAE) buffer by liquefying 0.9 g of agarose powder. The conical flask was then placed for 40 seconds in a microwave oven to heat the crude powder

and then cooled down. 4µl Ethidium bromide was added to 1.5% agarose solution after cooling and poured off to gel casting tray which was already affixed with the appropriate comb and get until solidifications. 1X TAE buffer was also used as a running buffer in the gel tank. The nested PCR product was blended with a 6X loading dye and loaded into the wells of the gel. 100bp DNA gene ruler (Thermo scientific) was loaded in a separate well for comparison of the size of amplified PCR products. After that the gel was run at 90V for 30mins, once the gel electrophoresis process completes gel was observed in UV trans-illuminator for visualization of amplified DNA.

Table 7: Components for gel electrophoresis procedure.

S. No.	Components (1.5%) gel	Amount
1	1 x TAE buffer	60 ml
2	Agarose	0.9 g
3	Ethidium bromide	4 µl

3.11 DNA extraction from agarose gel

The amplified fragment (Excised from the gel) was purified using Gene JET Gel Extraction Kit (ThermoScientific). The target DNA gel fragment was excised with a smooth razor blade and put in a pre-weighted autoclaved microfuge tube. Digital balance was used weighing the excised gel. After that the binding buffer was added to the tube as 1:1 ratio (weight: volume) i.e., 50µl Binding buffer was added to 50mg of gel slice and heat up the gel slice and binding buffer mixture at 60ºC for 10 min using heat block. Then transferred up to 800µl solubilized gel solution to the column and centrifuge for 1 min on 12000 rpm. After discarding the flow through, 100 µl more binding buffer was supplemented for sequencing purposes to the column. The mixture was centrifuged again on 12000 rpm for 60 seconds and discarded the flow through. 700µl Wash Buffer was added and centrifuged for 1 min on 12000 rpm and discard flow through. The empty columns were then centrifuged for 1 min on 12000 rpm to eliminate remaining wash buffer. The column was subsequently transferred to a sterile microfuge tube and 35 µl Elution buffer was added. This was centrifuged on 12000 rpm for 1min, column removed, and purified DNA fragment stored for further processing at -20 °C. For confirmation, 5 µl of obtained DNA was again run on agarose gel (1.5%) and visualized via UV trans-illuminator.

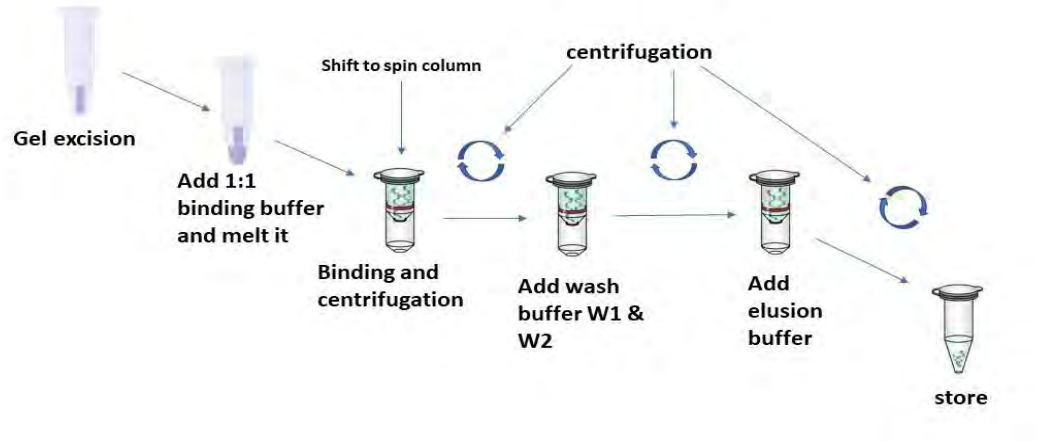


Figure 10: Gel purification procedure of amplified PCR products

3.12 Sequencing of influenza N gene (partial)

Sanger sequencing of the gel purified PCR product was done with Neuraminidase gene specific internal primers separately (forward and reverse). The sequencing results analysis was done through sequence alignment software called BioEdit. Nucleotide sequences obtained were in the form of ―FASTA" format for further analysis.

3.13 Blast Analysis

Basic local alignment search tool (Blast) (http://web.ncbi.nlm.gov/blast) was used to perform homology analysis of the query sequence (QAU-AS1, QAU-AS2) with the known Influenza nucleotides sequence present in NCBI GenBank.

3.14 Phylogenetic analysis

For the homology of the current sequences of the N gene with other sequences of the influenza of Pakistan as well as sequences from the rest of the world were search in the NCBI GenBank through nucleotide BLAST. All the available sequences were aligned through MEGA 11 software package (Tamura et al., 2021). The phylogenetic tree was constructed. Using maximum-likelihood approach the evolutionary history was estimated.

CHAPTER 4

RESULTS

4 Results

During the winter season of 2022-2023 samples were collected randomly from patients having flu like symptoms in Islamabad. Total of 25 samples were collected mostly from the students at Quaid-i-Azam University, in which only one was influenza negative and rest of 24 samples were influenza positive. All of the patients included in this study were adults having age ranging of 23-42 in which 20 were males and 5 were females. All the samples were collected in different timespan throughout the winter season.

4.1 Influenza detection through molecular method

Out of 25 swab samples 24 were qualitatively tested positive by M gene specific RT (reverse transcription) PCR M gene region of 229bp was amplified using M-229L as forward primer and M-229U as a reverse primer. The amplification was further confirmed by running the amplified product blended with loading dye, 1.5% agarose gel stained with Ethidium bromide Gel electrophoresis shows prominent amplified fragments located slightly above the 200bp compare with 100bp DNA ladder as shown in Figure 11. So, it is confirmed that those fragments were of 229bp in size.

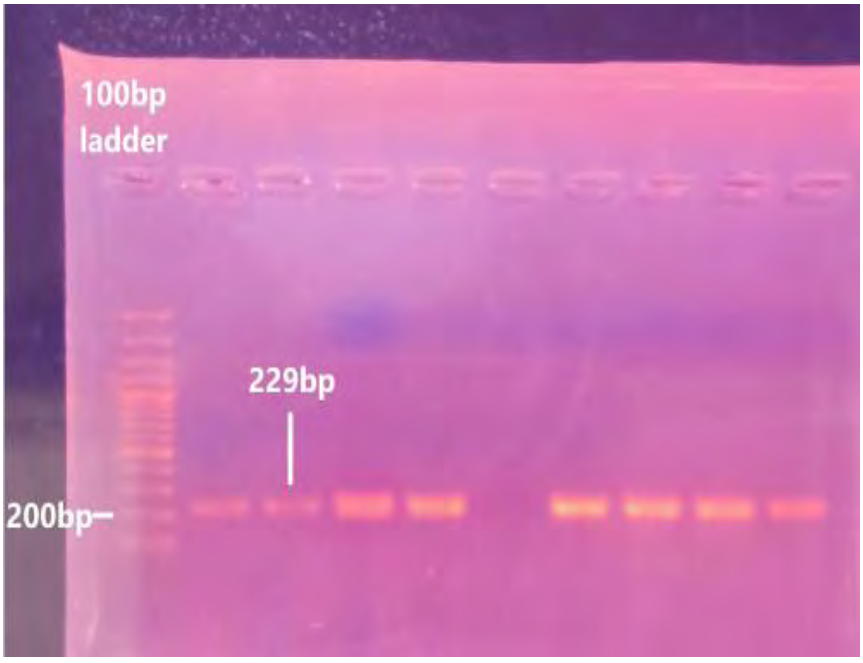


Figure 11: Gel electrophoresis and visualization of amplified product. Representing results of 10 samples (A1 to A10) in which A5 shows negative results.

4.2 Identification of subtype of Influenza A virus

In order to identify the subtype of influenza A, ten sets of oligonucleotide primers were obtained from previous studies. The first set contain N1, N2, N6, N8, N9 and the second set containing H1, H5, H6, H7, and H9.

4.3 Identification of viral N gene.

PCR reactions were performed by applying all the available set of primers to identify the NA subtype. In all those samples N1 amplified a fragment of its relevant size, and the rest of other primers did not show any amplification as shown in Figure 12. Product size of amplification of N1 was 329bp. The amplification and its fragment's size were confirmed through gel electrophoresis. As shown in Figure 12 (A) the amplified fragments were slightly above the 300bp of DNA ladder sequence.

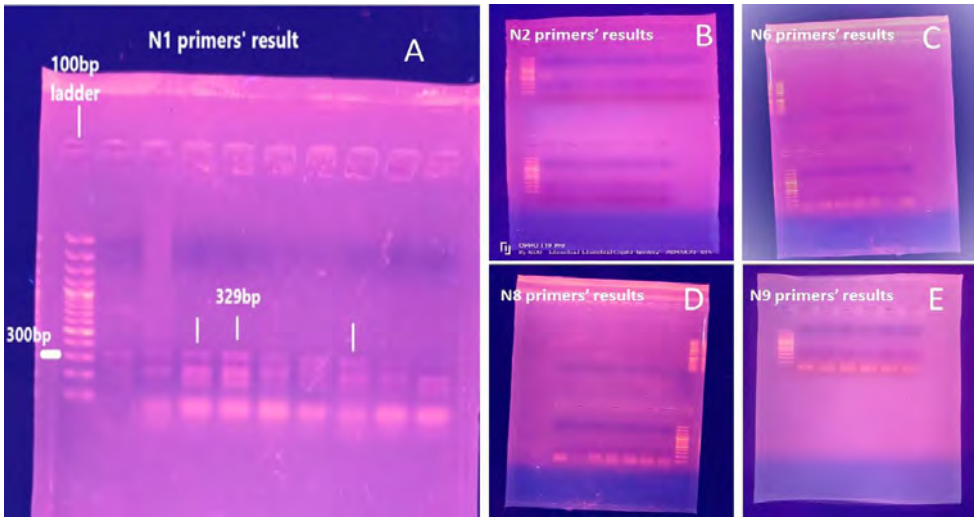


Figure 12: (A) represents the amplified fragment of N1 gene (B) represents the PCR result of N2 primers, (C) represents the PCR result of N6 primer, (D) represents the results of N8 primers, (E) represents the results of N9 primers. All the PCR products are with comparison to 100bp DNA ladder.

4.4 Identification of viral H gene.

For the identification of specific HA subtype of influenza A viruses, total of four oligonucleotide primers were designed and applied on each sample to confirm the exact recently circulating subtype. After the confirmation of N1 we were halfway behind our accurate results. By applying all four primers only H1 amplified a fragment of its relevant size of about 500bp but the results of gel electrophoresis were not so satisfied means that amplified fragments were not so clear. Gradient PCR reactions were carried out to optimize

the condition for HA gene amplification. At 56°C annealing temperature, sound and clear fragments were observed. Three samples were processed for amplification of HA gene with H1 primer at that optimized annealing temperature PCR product was run on 1.5% agarose gel stained with Ethidium bromide along with 100bp DNA ladder. Gel electrophoresis results are shown in Figure 13.

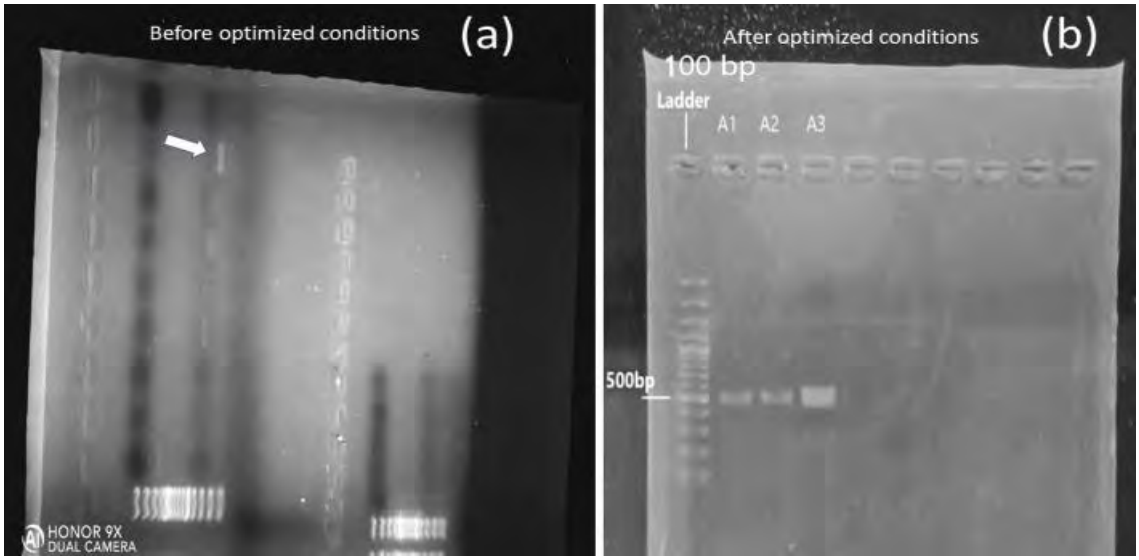


Figure 13: Represents results after gel electrophoresis. (a) shows weak amplified fragment of H1 product before optimized conditions. (b) shows clear and prominent bands parallel to its relevant size of 500bp.

4.5 Phylogenetic analysis.

Each sample after amplification was purified from excised gel using gel extraction kit and was further processed for Sangers sequencing. BioEdit software was used for refining sequence. In current study the obtained NA gene sequences were further analyzed through computational approaches.

For understanding the genetic variability, sequencing of the mentioned gene of Influenza A samples was performed. A total of 40 reference sequences of the H1N1 subtype have been acquired from NCBI database. Maximum likelihood Method was used for finding the evolutionary history Results are shown with the neighbor joining tree. Next to the branches, where the related taxa have been grouped together, is a percentage of trees. 42 nucleotide sequences were included in this valuation. Software package used for the evolutionary analysis was MEGA 11 (Tamura et al., 2021). The study demonstrates that influenza A NA gene isolates sequences of the current study (QAU-AS1) make a cluster with

previously reported sequence from Washington, and Nevada (MK716148, MK399163) while QAU-AS2 make a cluster with previously reported sequence from South Africa (MN716554), which depicts that they might have been originated from above mentioned sequence recently. They could be thought out as emerging viral isolates. The taxa of current study viral isolates are labelled as red, while other isolates of Influenza A NA gene, mostly from South Africa, and Washington and other regions are presented in black colour as shown in the Figure 14.

Figure 14: Phylogenetic tree of the NA gene nucleotide sequences of studied isolates (n=2) with the other reference sequences (n=42) from different countries of the word. The current study viral isolates are named (QAU-AS1, QAU-AS2) labelled as red, while reference sequences are represented in black colour.

CHAPTER 5
DISCUSSION

5 Discussion.

The significance of influenza as an emerging and re-emerging illness has grown in the last three decades, owing to the introduction of new and possibly deadly strains of fowl and swine origin. The number of fatalities of this pandemic influenza is significant in terms of sickness, death, and economic cost, and there is the possibility of significant societal disturbance. Given Pakistan's complex physical topography and accompanying climatic variations, ongoing surveillance is critical for assessing illness patterns and the healthcare system's burden. An adequate understanding of influenza virus circulation and seasonal patterns using laboratory-based surveillance is required for the efficient and effective allocation of scarce resources for pandemic preparedness.

Spontaneous influenza cases were found in Pakistan throughout the summer of 2022, from April to August, while seasonal influenza viruses persisted in circulation. during the winter, from October 2022 to February 2023, with a peak in December. This trend is similar to that of tropical nations such as Singapore, Thailand, and Northern Vietnam, where influenza viruses are present all year round in contrast to temperate locations, where transmission shows a clear seasonality (James et al., 2007; Simonsen, 1999). Yet, variations in environmental parameters including humidity, sun radiation, and temperature have been associated with seasonal influenza (James et al., 2007). In Pakistan, a country in the temperate zone, these elements may possibly have an impact on the influenza virus's distribution. Similarly, for temperate regions, annual fluctuation in influenza prevalence rate has been observed (Badar et al., 2013; Mez et al., 2017). Our subtype data indicates influenza circulation tendencies that are comparable to those in other temperate regions, such as Northern China and South Africa where significant influenza transmission rates have been reported throughout the winter (Hirve et al., 2016; Shu et al., 2010). Therefore, we draw the conclusion that throughout the research period, influenza circulation in Pakistan had a remerging trend in both temperate and tropical regions. By regularly tracking the incidence of Influenza related illness and mortality, looking into odd respiratory epidemics, and characterizing current influenza viruses, local and regional public health professionals may increase pandemic preparation.

Type A is particularly important in the development of any epidemic or pandemic condition. Because it evolved more quickly and has a wider variety of hosts than the other two influenza virus types (Dawood et al., 2009). The 1918 Spanish flu (H1N1 influenza

virus) pandemic killed a significant number of people globally, including 6,75,000 people only in the USA (Johnson and Mueller, 2002). A completely new variant of the influenza A virus (H1N1) emerged in Mexico in 2009 as a consequence of the reassortment of several flu viruses (Scalera and Mossad, 2009). About a decade ago, H1N1 had surpassed all widely circulating seasonal flu viruses (Smith et al., 2009). Approximately 43 to 89 million individuals were infected by the 2009 (H1N1) pandemic, which also killed 8,870 to 18,300 people and resulted in several regional epidemics in other nations, including Pakistan (Shrestha et al., 2011). In comparison with seasonal flu, pandemic viruses frequently infect healthy people who are young or old, as well as those who have comorbid conditions (LaRussa, 2011). The influenza A virus subtypes H3N2/H1N1, influenza B, as well as the new (H1N1) pdm09, has been recognized as the most widely circulating viruses by the WHO. The infection often strikes temperate regions throughout the winter (Hannoun, 2013). The objective of the current. study was to characterize the subtype using genetic approaches that were common in Islamabad during the winter of 2022–2023.

The flu viruses are ubiquitous throughout the country and infect people of all ages (Badar et al., 2013; Khalil et al., 2017). The threat of influenza is similar in Pakistan, as acute respiratory tract infections represent one of the major causes of mortality in children under the age of five (20-30%) (Khan et al., 2004). In Pakistan, the flu is a significant cause in the rise of self-medication (Nazir and Azim, 2017). People are more inclined to follow health guidelines if they are given precise and readily available information from healthcare authorities about the malady and effectively responding, as well as if they are kept informed that the outcomes of not doing so are likely to be severe. This will help prevent the spread of the disease outbreak (Abalkhail et al., 2017). In 2008, Pakistan built an influenza monitoring system that recorded data from eight sentinel locations (high burden tertiary level) spread across six regions. But since no thorough analysis of the surveillance data has really been carried out Previous research in Pakistan examined the molecular epidemiology and pathophysiology of the 2009–2010 pandemic's influenza A(H1N1)pdm09 viral infection (Nisar et al., 2014). In just four sentinel sites, different research examined data from the influenza monitoring program from 2008 to 2011 (Badar et al., 2013). Therefore, this study was conducted to identify newly circulating subtypes that may contribute to the understanding of influenza A epidemiology.

This study examines the NA gene of influenza A virus circulating in Islamabad, Pakistan. Those sequences resembled the influenza A vaccine strain (H1N1). Nevertheless, more antigenic analysis is required to evaluate the properties of sequenced viruses, particularly those that have modifications at the hemagglutinin's antigenic and glycosylation sites. Our research confirms the genetic variation of the influenza A(H1N1) pdm09 viruses and highlights the value of routine molecular surveillance for the efficient control of influenza pandemics and epidemics.

Conclusion

In Pakistan the prevalence of influenza A virus is still increasing and there is no such effective surveillance system in Pakistan. It is very necessary to identify the emerging or recently circulating subtype of influenza. In order to prepare ourselves for upcoming epidemic or pandemic. In the current study, subtype was identified and NA gene (partial) of influenza A was amplified, sequenced and its phylogenetic analysis conceals that the current sequences (isolate QAU-AS1 and QAU-AS2) grouped together with (MT639158, MT554136, MK849857, MK716148, MK399163) and shows 100% similarity with H1N1. It demonstrates that the current vaccination is still effective to overcome the prevalence of influenza in Pakistan. Further studies are required for comprehensive surveillance throughout the country.

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