Molecular Detection of Bats Associated Influenza Virus

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

DECLARATION OF ORIGINALITY

I hereby declare that the work **"Molecular Detection of Bats Associated Influenza Virus"** 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 ~~"c~~opy 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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Dedication

I dedicated this work, especially to my uncle "Dr. Muhammad Ajmal" my parents, and my charming Family

Thank you for your being

My best friend, my comfort when things go wrong,

The ones who encourage me, believe me,

Change my "~~w~~eak" 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

RNA	Ribonucleic acid
PB	Basic polymerase
PA	Acidic polymerase
HA	hemagglutinin
NP	Nuclear protein
NA	neuraminidase
NS	Non-structural protein
M	Matrix protein
PCR	Polymerize chain reaction
SARS	Severe acute respiratory syndrome
ICTV	International committee on taxonomy of Viruses
IAV	Influenza A virus
mRNA	Messenger ribonucleic acid
vRNA	Viral ribonucleic acid
RdRp	RNA-dependent RNA-polymerase
WHO	World health organization
AIV	Avian influenza virus
MERS	Middle East Respiratory Syndrome
RBC	Red blood cell
FAO	Food and agriculture organization
OIE	Office international des epizooties
USAID	United States Agency for International Development
USDA	United states department of agriculture
EU	European Union
DFID	Department for international development
BBSRC	Biotechnology and biological sciences research council
DANIDA	Danish International Development Agency
swIAV	Swine influenza A virus

HIAVs	Human influenza A virus
HLA-DR	Human leukocyte antigen-determinant region
MHC	Major histocompatibility complex proteins
RNP	Ribonucleoprotein
VTM	Viral transport medium
BCL	Biosafety cabinet class level
cDNA	Complementary deoxyribonucleic acid
Uni	Universal
dNTP	Deoxynucleotide triphosphate
ddH2O	Double distilled water
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris acetic acid
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
PDB	Protein drug data bank

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Abstract

As the second-most diverse group of mammals, bats are found on every continent except Antarctica and have been implicated in the spontaneous transmission of several zoonotic virus infections such as Influenza Virus. Bats-associated influenza viruses have been reported from different countries like China, Brazil, Egypt, South Africa, South America, and Central America etc. However, there is no study from Pakistan involving bats-associated influenza viruses. For this purpose, bat guano was collected from the natural surroundings in various areas of district Swat province Khyber Pakhtunkhwa, Pakistan. The samples were processed for viral RNA extraction, followed by viral cDNA synthesis which was subsequently processed for amplification of selected genome region (M gene, partial) of influenza virus. The resulting amplified M gene region were assessed for nucleotide sequencing through Sanger sequencing method. Evolutionary relationship of the amplified sequences of M gene of our isolates QAU-HA2 clustered with a previously reported sequence from Russia (Accession no. KF462347), isolate QAU-HA5, QAU-HA3, QAU-HA4 and QAU-HA9 clustered with sequences reported from Netherland (Accession no. MF145856). We suggest a more detailed molecular study should be done for better understanding of Influenza A viruses circulating in bats.

Keywords: Bats influenza; M gene; Phylogenetic analysis; Arc GIS 10.8 version.

CHAPTER 1

INTRODUCTION

1 Introduction

Bats serve as potential transmitters for a variety of infectious agents, including, SARS-CoV, Ebola, Nipah, Hendra, Influenza A and Marburg viruses (D. Hayman et al., 2013). The possibilities for either direct or indirect interaction with people and other livestock, as well as the widespread distribution, great species variety, and substantial population numbers of bats, highlight the importance of trying to properly recognize the dynamics of bat-borne viral infections (Kandeil et al., 2019). In addition, it is estimated that around 75% of all newly identified infectious diseases that affect or endanger the health of humans are zoonotic (Field, 2009).

Most of these viruses have either accidentally or intentionally reached people after leaking into animal reservoirs. Risk issues like international travel, trading, growth in agriculture, deforestation dispersion, and urbanization raise the connection or the degree of interaction among individuals, wildlife, and domestic populations, which increases the likelihood of spillover incidents.

Since shorebirds and ducks were thought to be the sole habitats for influenza A viruses (IAVs), (9 neuraminidase and 16 hemagglutinin) variants of IAVs have previously been identified in these species. But in the years 2012 and 2013, there were two additional subtypes found (H17N10) and (H18N11), was detected in the small, yellow-shouldered bat (*Sturnira lilium*) and the flat-faced fruit-eating bat (*Artibeus jamaicensis*) individually [8, 9]. Only phyllostomid bats as host for these subtypes in South America (Rimondi et al., 2018).

Bat IAVs have been proven to be vulnerable to infections by classical IAVs, even though their zoonotic potential has not yet been fully demonstrated and is still a subject of ongoing research. In a recent investigation, 30% of the examined *African frugivorous bat* had antibody proof of IAV H9 variant infections (Freidl et al., 2015). It is possible that the repeated replication of Influenza A virus in an unfamiliar host species, which sparks the emergence of new viral variations, is a significant driver of IAV genetic variation (Poole et al., 2014).

Furthermore, the cave-dwelling *R. aegyptiacus* is known to serve as a host for a variety of infections throughout Africa, including the Marburg virus, coronaviruses, lyssaviruses, and influenza viruses (Pawęska et al., 2020; Tong et al., 2013). *R.*

aegyptiacus favours tropical and subtropical areas, having a geographical range that stretches from northern Africa to sub- Saharan Africa and from south-west Asia to Pakistan and Iran (Benda et al., 2011; Lučan et al., 2016). More cases of flu Bats have been identified as possible hosts for new influenza viruses after virus discovery and characterization from various bat species (Rademan, Geldenhuys, & Markotter, 2023).

Influenza A viruses are significant for the public's safety because they have contributed to prior emergencies and pose a resurgent risk. Flu viruses can cause substantial death and disability in populations that are susceptible when they cause periodic epidemics and seasonal illnesses (Uyeki et al., 2019). Influenza based on the exterior peptide‘s neuraminidase and hemagglutinin; virus types A are divided into categories. 16 hemagglutinin and 9 neuraminidase categories were recognized by the ICTV prior to 2011 (Rademan et al., 2023).

Moreover, IAV is categorized as belonging to the *Orthomyxoviridae family* (orthos, which means "standard, accurate," and myxa, which means "mucus" in Greek). These are single-stranded RNA virus having a segmented genome that is negative-sense, and enclosed. Both messenger (mRNA) and complimentary RNAs are synthesized from the viral RNAs (vRNA), which serve as templates (Cheung & Poon, 2007; Wu & Wilson, 2020). This family has been divided into four genera; influenza A, B, C, and D individually (Cheung & Poon, 2007).

The genomic composition of influenza A virus includes, PB1 gene, PB2 gene, PA gene, HA gene, NP gene, NA gene, M gene and NS gene segments, which are numbered one through eight (Bouvier & Palese, 2008). IAVs start an infection by attaching their surface glycoprotein to sialic acid receptors on the glycoproteins of the host cell. Sialic acids were once believed to be the universal receptors for all influenza virus strains, promoting interspecies transmission. Interestingly, nevertheless it was shown that the new world bat influenza strains H17 and H18 lacks this characteristic and cannot recognize these sialic acid receptors, but molecules of the MHC-II class, which are necessary for cell entrance (Ciminski, Pfaff, Beer, & Schwemmle, 2020).

Due to the presence of highly conserved regions, the PB1 and M genes were chosen as candidates for molecular monitoring studies. The major focus for identifying new viruses of influenza A form bats was the PB1 gene. The PB1 gene was the main candidate for identifying new influenza strains. Previously, IAV from bats with

mutations in the M gene demonstrated decreased assay effectiveness and difficulty in identifying novel influenza strains (Duh & Blaţ ič, 2018; Tong et al., 2013).

The discovery of novel bat flu viruses is not unexpected given that bats make approximately 24% of all identified mammal species (Smith & Wang, 2013), and are known to act as natural hosts for a variety of dangerous pathogenic viruses (Brook & Dobson, 2015; Memish et al., 2013). According to some estimates, bats can harbour greater genetic variation of the flu virus than any other mammal and bird species together (Tong et al., 2013).

Our research sought to examine the bats associated with influenza virus from its fecal samples. Moreover, Polymerase chain reaction (PCR) were performed to confirm the positive influenza samples and to explore the Matrix protein (M) gene of influenza isolates for nucleotides sequencing, and its phylogenetic analysis.

1.1 Aims and Objectives

- To detect influenza virus from bats guano in Pakistan.
- To perform nucleotide sequencing and phylogenetic analysis of M gene of influenza virus circulating in bat-population.

CHAPTER 2
REVIEW OF LITERATURE

2 Review of literature

2.1 Zoonosis

The name –zoonosis" comes from the ancient Greek words –zoon" which mean (animal) and –nosis" mean (disease). Zoonosis is a term used by the World Health Organisation (WHO) to describe any illness or disorder that can spread spontaneously from vertebrates creatures to humans or from people to animal (M. T. Rahman et al., 2020). The genesis and spread of several infectious diseases are significantly influenced by individuals, livestock, and the natural environment (Thompson & Kutz, 2019). These pathogens include viruses, bacteria, fungi, protozoa, and parasites that are present in nature but not only in humans (Taylor, Latham, & Woolhouse, 2001). Majority of contagious diseases that impact people have animal origins. The prevalence and seriousness of zoonotic illnesses that impact humans have been rising from decades to decades (K. E. Jones et al., 2008). Over 70 percent of these pathogens evolved from wildlife populations, according to the analysis "Asia Pacific regions plan to combat rising illnesses 2010, which predicted that zoonotic illnesses make up about 60% of new infections impacting humans (M. T. Rahman et al., 2020). Zoonoses pose a serious risk to human health and are potentially fatal, making them a major public health problem. Globally, the 13 most widespread zoonoses affected poor livestock workers in nations with low or middle incomes the most, and they are also responsible for approximately 2.7 million human fatalities annually as well as 2.4 billion instances of disease (Grace et al., 2012). Furthermore, Zoonotic illnesses have a terrible effect on the economic growth, income, and health of both humans and animals. With varying levels of mortality and morbidity, financial damage, and loss of productivity, zoonotic diseases brought on by viral infections have dominated the global disease landscape for the previous two to three decades. For examples, the currently underway coronavirus disease 2019 (COVID19) outbreak brought on by the *SARS coronavirus-2* has been inappropriately categorized as a zoonotic illness due to no animal-based host has yet been identified. It is suggested that COVID19 be labelled as an infectious illness of possible animal source (Haider et al., 2020).

By 2050, the number of people on the planet is projected to rise from 6.5 billion in 2008 to 9.2 billion (Fløttum & Dahl, 2012). With expected rise in demand for food of up to 50% by 2030, notably for dairy and meat products, this offers enormous

difficulties for the farming industry generally (Delgado, Rosegrant, Steinfeld, Ehui, & Courbois, 2001; P. G. Jones & Thornton, 2009). Two agricultural production techniques are being used to satisfy this demand, but they each carry their own dangers related to the introduction and spread of Zoonotic illnesses. One is very low-level poverty farming of livestock, which is practiced in rural homes in the world‘s poorest regions and focuses mostly on raising chickens, sheep, and goats. Many times, these animals remain in poor surroundings with no focus on illness prevention, shelter, or supplemental feeding (Tomley & Shirley, 2009). The animals, who carry a heavy load of endemic diseases, interact often with human and other species of livestock, and they may also come into touch with a wide range of exotic animals. These circumstances encourage closer interactions between people and animals and raise the risk of zoonoses spreading (Alexander, 2007).

2.2 Classification of Zoonoses

Numerous microorganisms are responsible for zoonotic illnesses. Zoonoses are divided into groups according to their etiological causes: bacterial zoonoses, viral zoonoses, Zoonotic caused by parasites as well as zoonoses caused by fungi (Schaechter, 2009). These illnesses are especially significant considering recently developed infectious diseases in humans because most of these are zoonotic in nature (Cleaveland, Laurenson, & Taylor, 2001).The primary zoonotic illnesses are listed in Table 1 along with their main signs, animal hosts, and etiological factors.

Table 1: : The most prevalent zoonotic illnesses, etiological factors, their hosts, and the main signs in individuals (M. T. Rahman et al., 2020).

Infections	**causes of Animal**	**Hosts**	**signs, related systems, or organ**
Bacteria Zoonosis			
Anthrax	*Bacillus anthracis*	mink, sheep, pigs, Cattle, elks, bison, goats, horses	System of respiration, GI tract, or skins
Tuberculosis	*Mycobacterium microti, bovis,* and *caprae*	Bison, deer, bears, camels, sheep, cattle.	Marrow in the bones and the respiratory system
Brucellosis	All the *Brucellaes*	Dogs, sheep,	Weakweight,

	include,fetalis,meleten sis, canis and *suis*	swine, goats, and calves	discomfort in the back, stiffness in the joints, and a high temperature that is typically severe in the late afternoon
Bubonic plague	*Yersinia pestis*	Prairie dogs, wood rats,groundsquirrels, rock squirrels, mice	Flu, shivers, stomachache, constipation, nausea, and blood from an incision in the skin.
Glanders	*Burkholderia mallei*	Equine, donkey, and mules	Flu, shivering, migraine, discomfort in the muscles, pain in the chest
Leprosy	*Mycobacterium leprae*	Rodents, rats, felines, and monkey	Dermal blemishes
Leptospirosis	*Leptospira interrogans*	Dogs used as pets are among the wild and domesticated animals.	Red eyes, yellowing of the skin, stomach-ache, and temperature.
Tularemia	*Francisella tularensis*	Bulls‘ snakes as well, natural rats, bears, dog and cat	Cough diarrhoea, and discomfort in the joints.
Arcobacter infections	Three types of bacteria are *Arcobacter:Butzleri, Skirrowii* and	Sheep, swine, chicks, and cows	Vomiting, a high temperature, and discomfort in the stomach

	Cryaerophilus,		
Actinomycosis	*Actinomyces bovis*	Animals such as cattle, sheep, horses, swine, and cats	lymphatic node, soft tissue, the epidermis and abscesses inflammation
Bordetellosis	*Bordetella bronchiseptica*	dog and Cat	pulmonary condition
Lyme disease	*Borrelia burgdorferi*	cattle, pets, and cat	Scalp itchy, dermatitis migrans, a high temperature, and headache
Campylobacter enteritis	*E. coli* and *Campylobacter jejuni*	swine, pets such as cats and dogs, mink, rabbits, lambs, and poultry	intestinal disease
Clostridioides difficile infection	*Clostridioides difficile*	mammals, donkeys, and cows	diarrhoea, pseudoembryonic colon
Helicobacter infection	*Pullorum* and *suis* strains of *Helicobacter*	Porks and chickens	stomach ulcers
Vibriosis	*Parahaemolytic Vibrio*	Farming animal	Small intestine inflamtion
Zoonoses virus			
Rabies	*Rabies virus*	Animals such as wolves, skunks, bunnies, cats, dogs, bats	the body‘s CNS
Newcastle disease	*Paramyxovirus*	Chickens and avian wildlife	ophthalmitis
Avian influenza	*Influenza A virus*	Wild birds, cats, dogs, swine,	diarrhoea, respiratory

		cattle, dolphins, seals, and ducks	infections
Rift Valley fever	*Rift Valley fever virus*	Camel species, calves, goats, sheep, buffaloes	Flu, joint discomfort, muscular soreness, and headaches.
Ebola virus disease	*Ebola virus*	Apes, gorillas, chimpanzees, monkeys	Flu, severe fatigue, aching muscles, migraines, throat pain, bleeding, nausea, vomiting, diarrhoea, kidney damage
Marburg viral disease	*Marburg virus*	apes and mammals such as, bats	bleeding, fever, discomfort in the muscles, diarrhoea, discomfort in the abdomen.
Hantavirus infection	*Hantavirus*	White-footed mice, deer mice, cotton, and rice rats	stomach issues, a raised temperature, fainting, shivers, and respiratory issues.
AIDS	*HIV*	chimps and monkey	Immunosuppression, signs like the flu, a high body temperature, an irritation, sweating during the night, aches and pains in

			the muscles
Monkey pox	*Monkeypox virus*	Various species of primates, stolen rats from Gambia‘s.	temperature and blisters on the skin caused by the chicken pox.
Zoonotic parasitism			
Trichinellids	*trichinosis species*	Mice, rodent, dog, cat, and various other wild creatures	Pain in the abdomen, vomiting, diarrhoea, and other symptoms of digestion.
Visceral larva migrans	*Toxocara canis* and *cati,* and *Ascaris suum* are among the species of *Baylisascaris procyonis.*	Birds, emus, cats, chinchillas, prairie dogs, weasels, and woodrats are among the animals that live there.	Stomach discomfort, high temperature, shortness of breathing, and signs of digestion
Cutaneous larval migrans	*Brazilian encylostoma*	Cat, Dog	tissue beneath the skin.
Hydatidosis	*Granulomatous Echinococcus*	Sheep, goat, and shepherds‘ dog	stomach pain, cysts of the hydatid lining in the the hepatocyte the lungs, skeletons, bladder.
Cryptococcosis	*Neoformans Cryptococcus*	Cows, horses, and sheep, goats, pet dogs, wild creatures	issues with the lungs, a high temperature, nauseousness, and diarrhea.
Fascioliasis	*Hepatic* and *enormous*	ruminant species	Severe pain in the

	fasciolia	such as cattles, sheeps, and goat	abdomen, a high temperature, sickness, an enlarged the hepatocyte, irritation of the skin.
Zoonotic fungus			
Infection with tinea	*Microsporum* and *Trichophyton species.*	All creatures, including dog, cat, sheep, calves, and goat	Dermal blemishes
Aspergillosis	Many different species of *Aspergillus.*	Each pet and bird.	Breathing issues
Histoplasmosis	Capsular variant of *Histoplasma pneumonia*	Rodents, animals such as rabbits‘ puppies, and cat	Flu, a productive cough, pain in the chest, decreased weight, and bleeding disorders are frequently
Blastomycosis	*B. dermatitidis*	most frequently found in cats and dogs, and less frequently in cattle, rabbits, elk, foxes, and marine lions.	Seizures, verrucous blisters, chronic pneumonia, meningitis, a high temperature, and discomfort.
Coccidioidomycosis	*Coccidian immitis* and *posadasii*	Cattle, pork, pets, and other ruminant species	Flu, dermatitis multiform, erythema nodosum, arthritis, discomfort in the chest, and dry

			cough are all signs of an allergic reaction.
Cryptococcosis	Neoformis of the *cryptococcus*	wild creatures, livestock, horse, sheep, goat and dog,	Meningitis, fever, malaise, headache, neck stiffness, photophobia, cough, nausea, and vomiting

2.3 Classification on the Base of Transmission

Anthropozoonoses, Amphixenoses, Zooanthroponoses, and Euzoonoses are some of the words used to describe zoological diseases in the past (Hubálek, 2003). Animal diseases known as ―anthropozoonoses," such as rabies, can infect humans. Zooanthroponoses is the term used to describe diseases that people spread to animals, such as tuberculosis that affects cats and monkeys. Diseases known as amphizoonoses, such as staphylococcal disease, can spread in any route (from person to animals and from animals to person). Most zoonotic illnesses have bacterial etiologies. According to estimates, of the pathogens that are zoonotic and derived from cattle, roughly 42% are bacterial, 22% viral, 29% parasitic, 5% fungal, and 2% prion in source (McDaniel, Cardwell, Moeller, & Gray, 2014). Additionally, zoological infections are known to be caused by both DNA and RNA viruses; However, RNA viruses are more commonly associated with zoonosis when compared to DNA viruses (Bae & Son, 2011). Both direct and indirect animal-to-human transmission of pathogens is possible. Direct zoonoses are conditions when a disease spreads from an animal to a human through a medium like the air (Mortimer, 2018).

2.4 Animals as Reservoirs of Viral Zoonoses

The abundance of infectious microorganisms that can cause disease, the term ―natural reservoir" has been used in a variety of ways over the years with no clear definition. An infectious pathogen's ―natural reservoir" is a group of organisms or the environment in which it normally exists and propagates, or upon which it predominantly requires for existence, and from which it is spread to the desired population (Haydon, Cleaveland, Taylor, & Laurenson, 2002). Infectious illness

natural reservoirs the environments in which an agent typically survives, develops, and reproduces. The reservoirs may target the same species or different species, contain one or more distinct species, and contain vector species (Haydon et al., 2002). Numerous viruses are stored in animals where they can occasionally cross species and attack humans. Animal reservoirs are made up of domesticated and wild animals that have been exposed to infections, and many zoonotic diseases can be linked to these species (Tomori and Oluwayelu, 2023).

For instance, in nature, *lyssaviruses* (such as rabies) are linked to particular mammals that serve as reservoir hosts, such as bats and carnivores (Tomori & Oluwayelu, 2023). while the *Ebola and Marburg viruses* natural reservoirs are still unknown, however bats are the top candidate for both viruses maintenance in exposed small animals (Koch, Cunze, Kochmann, & Klimpel, 2020). It is well known that animals play important roles in the spread of pathogenic viral illnesses to humans, especially in the case of those who are exposed due to their occupations, such as veterinarians, butchers and abattoir workers, livestock farmers, investigators, animal owners (through scratching or biting of the owners of indoors pets), zookeepers, and hunters (Sick, Beer, Kampen, & Wernike, 2019). Some of the most common viral zoonoses are described in Table 2 along with information on their geographical distribution, hosts of animals, and modes of transmission.

Table 2 : The primary infectious zoonoses' distribution across the globe, hosts of animals, and means of propagation are shown (Sick et al., 2019).

Geographic distribution	**Viruses**	**Animal reservoirs**	**Transmission**
worldwide	*Rabies-virus*	all creatures with warm blood, but notably dogs	Virus-carrying animal's bite
	Hanta-virus	Rats	Aerosol rat urine or faces, spit or a rodent bite that has the infection.
	Yellow fever virus	Non-human primates	biting of an infectious mosquito, mainly an Aedes species

	Chikungunya virus	apes, birds, small mammals, and rodents	biting of an infectious mosquito, mainly an Aedes species
	Crimean-Congo hemorrhagic fever virus	Animals such as kept in the home (cows, sheep, and goat)	bites by infected ticks (mostly Hyalomma species); contact directly with the bloodstream or cells of affected ticks.
	West Nile virus	Birds	bites of an infected flies, mainly a Culex species vector.
Middle East and Africa	*Rift Valley fever virus*	Domesticated ruminants, including camels, goats, sheeps, and cattles	Infected animal bites (mostly Aedes species), interaction with sick animal fluids or tissues, and intake of unpasteurized milk are all possible sources of infection.
	Ebola virus	animals other than humans, fruit-eating bats, and porcupine.	exposure to sick animals‘ blood, saliva, organ, or other body liquids.
	Monkeypox virus	Rats, non-human primates	Interaction with respiratory waste, lesions on the skin, or body fluids of diseased people or animals, as well as polluted things.
	Marburg virus	Rousettus aegyptiacus, or fruit eating bats.	exposure to sick bat faeces, particles, blood, secretions, tissues, as well as surfaces and items that have been exposed to these fluids.

United states	viruses that cause *equine encephalitis*	Wild animals such as birds and horses‘ Venezuelan equine encephalitis virus	Bites from an infected mosquito Aedes species.
	Colorado tick fever virus	rats in the wild	Rocky Mountains wooden ticks (Dermacentor andersoni) that have been bitten by disease.
	Zika virus	Non-human primate	xenotransplantation, transplacental transmission, sexual contact, donation of blood and its components, the bite of a mosquito with an infection (most commonly an Aedes species)
Europe	*Tahyna virus*	Hares, foxes, hogs, and rodents are small mammals. Red deer, and wild boar are huge mammals.	Virus-carrying Aegypti vexans bites
	Tick-borne encephalitis virus	Mice and voles are small rodents. Domestic and wild mammals include sheep, cattle, and goats.	ingestion of unprocessed milk; Ixodes and tick bites that are infected
	Usutu virus	Wilde and domesticated birds (sparrows, owls and partridges, jays, doves,	bite from an infected mosquito, primarily a Culex

		and jayjays)	species.
Asia	*Influenza viruses*	wild ducks, chickens, pigs, cattle, pets, and bat.	direct touch with sick animals, exposure to settings contaminated by their faces, saliva, or respiratory secretions
	(SARS) coronaviruses	Many different domesticated and wild creatures (palm civets, bats, and pangolins, cats, dogs.	Interaction with dirty floors or fumes; exposure to droplets from sick people.
	Japanese encephalitis virus	Ducks and swine	Virus-carrying Culex species.
Australia	*Hendra virus*	horses, fruit eating bats.	contact to the tissues, bodily fluids, and/or excreta of sick horses
	Kunjin virus	wetlands birds	mosquito bite, particularly that of the Culex annulirostris, which is infected.

2.5 Major Viral Zoonoses of Wild and Domestic Animals

Domestic animals sometimes act as amplifying agents for infections coming from wild animals, and they play a vital part in spreading of many diseases to humans (Morand, McIntyre, & Baylis, 2014). 60 % or so of infectious diseases affecting humans are caused by vertebrates (Klous, Huss, Heederik, & Coutinho, 2016). As

more vertebrate animals have been domesticated, there has been an increase in direct human-animal interaction (Pearce-Duvet, 2006). Dogs, cats, horses, pigs, and other domesticated pets serve as reservoirs for the viruses that cause domestic zoonoses and can spread the diseases to individuals (Samad, 2011).

2.5.1 Rabies

Rabies virus, which is a member of the *Rhabdoviridae family,* is one of the most deadly zoonotic diseases in the world, between 30,000 and 70,000 people die each year (Blanton, Krebs, Hanlon, & Rupprecht, 2006). Although dogs are the primary rabies virus carriers, other wild animals such as cats and jackals also serve as rabies virus transmitters. For its safeguarding and management, secure and successful human vaccinations are readily accessible, and infection can be eradicated at the source by continuous mass immunization of reservoir populations (Hampson et al., 2009). which could subsequently become a significant source of disease spillover to their human hosts (Singh et al., 2001).

2.5.2 Avian influenza

Avian influenza is a chicken infection triggered by the type A influenza virus, which belongs to the genera *Alphainfluenzavirus* in the *Orthomyxoviridae family* (Tomori & Oluwayelu, 2023). It is one of the most serious public health issues to emerge from animal sources in recent years. In their native wild bird hosts, this virus are thought to be in an evolutionary stasis where the virus and the host get along (Gorman, Bean, & Webster, 1992). But given that they cause a disease that is detrimental to the industry's economy and the public health, the recent outbreaks of poultry sickness found in many Asian, European, and African countries are concerning (Control & Prevention, 1997). In 1997, the AIV A/H5N1 variant appeared in Hong Kong, where it caused substantial (75–100%) flock mortality in market chickens and six deaths out of eighteen instances in humans (Claas, de Jong, van Beek, Rimmelzwaan, & Osterhaus, 1998).

2.5.3 SARS and MERS Coronaviruses

Despite the fact that coronaviruses can infect both humans and animals with respiratory and gastrointestinal illnesses (Knipe et al., 2013), Prior to the *SARS* incident in Guangdong Province, China in 2002–2003, they were not thought to be

particularly dangerous to humans (N. Zhong et al., 2003). Alpha- and betacoronaviruses are prevalent in mammals, while gamma- and deltacoronaviruses exist in birds. Together, these four genera make up the *family Coronaviridae* (Woo, Lau, Huang, & Yuen, 2009). Previous research revealed that *SARS* and *MERS* coronaviruses evolved in bats (Lau et al., 2013; Lau et al., 2005) but spread to humans via subsequent amplification hosts, such as masked palm-spotted civets and dromedary camels, particularly (Alagaili et al., 2014). The transfer from bats to humans and the function of the secondary host in propagation are yet unknown,(Frutos, Serra-Cobo, Chen, & Devaux, 2020) despite the fact that coronaviruses are extensively distributed in wildlife. It was highlighted that domesticated animals might play significant roles as secondary hosts that help carry viruses from natural hosts to humans (Cui, Li, & Shi, 2019).

2.5.4 Nipah and Hendra Viruses

Two extremely dangerous infectious viruses (*Nipah and Hendra*) are members of the genus *Henipavirus*, which belongs to the *family Paramyxoviridae*. In humans, these viruses can cause catastrophic encephalitis with mortality rates fluctuating between 40% to 100% (Chadha et al., 2006). Nipah virus was discovered for the first time in Malaysia in 1999, where it has caused major illness in both humans and animals (Chua et al., 1999). Primary host of transmission to people are through contact with sick pigs (Chua et al., 1999). while there have also been reports of transmission from consuming food products polluted with *pteropid* fruit bats' saliva or urine (Luby et al., 2006), Cattle and other domestic animals were linked to Nipah virus related disease in the incidents in Bangladesh (Luby et al., 2009).

Furthermore, Hendra virus first appeared in two incidents of severe respiratory sickness in thoroughbred horses in *Hendra*, Australia in 1994. At the time, it was thought to be an *equine morbillivirus* where exposure to sick horses has led to human deaths (Murray et al., 1995). Pigs are prone to *Hendra virus* illnesses and may serve as intermediary hosts for human infection (M. Li, Embury-Hyatt, & Weingartl, 2010). Additionally, reports of dog Hendra virus infections exist (Kirkland et al., 2015).

2.5.5 Crimean-Congo Hemorrhagic Fever

A contagious zoonotic viruses illness spread by ticks that is native to, Asia, Africa, Eastern and Southern Europe is known as *Crimean-Congo hemorrhagic fever* belong

family *Nairoviridae* (genus *Orthonairovirus*) (Garrison et al., 2020). This virus is amplified by a range of domestic animals, including, camels, sheep, goats and cattle and is transmitted by ixodid ticks, which also act as the virus source and carrier (Bannister, 2010). Recently, researchers found viral-specific IgG antibodies in 24% of investigated cattle and 2% of examined goats, respectively. They came to the conclusion that these domestic species constitute a risk to the public health, especially for those who are exposed to them at work, including veterinarians, butchers, and agricultural workers (Akuffo et al., 2016).

2.5.6 Viral Zoonoses of Companion Animal, Pets, and Bird

A whopping 14-62% of pet owners let their animals inside their bedrooms, which could help zoonoses spread (Chomel & Sun, 2011). Due to the possibility for disease transmission, the widespread acceptance of pets and other companion animals has increased the risk to human life. As a result, many people are at danger of contracting a new zoonotic illness through pets, companion animals, and exotic birds and animals. Pets and companion animals are susceptible to a wide range of infectious illnesses (parasitic, viral, fungal and bacterial) (Halsby, Walsh, Campbell, Hewitt, & Morgan, 2014). The viral zoonotic illnesses linked to pet and companion creatures are another concern which includes hantavirus, influenza, rabies and monkey pox (Halsby et al., 2014).

Modern industrialized and developing nations both have large populations of birds including parakeets, sparrows, canaries, budgerigars and canaries (Boseret, Losson, Mainil, Thiry, & Saegerman, 2013). These hunting and elegant birds could spread viral zoonotic diseases like pet animals which includes Newcastle disease virus, fowl pox virus (Moro, Chauve, & Zenner, 2005). Via contact either directly or indirectly, these animals can transmit infections to humans, transfer might occur indoors, outdoors, at pet stores, hospitals, or other locations (Vanrompay et al., 2007).

2.5.7 Control of Zoonoses

The global community faces a major threat to its health from zoonoses, Approximately 58-61% of human illnesses are spreadable, with up to 75% being zoonotic (spread by animal) (Al-Tayib, 2019; Ng & Sargeant, 2013). A multi-sectoral strategy is necessary to guarantee successful control measures for zoonosis because it involves interactions between people, animals, and the environment (Aenishaenslin et

al., 2013). For zoonotic illnesses to be prevented and controlled, surveillance is essential. It can be exploited to identify early infection, afflicted people and animals, reservoirs, carriers, and endemic regions including the "hotspots". It is necessary to conduct surveillance on all potential infectious diseases sources such as pets, aquatic animals, exotic animals, birds, wild animals, companion animals and rodents (Van der Giessen, van De Giessen, & Braks, 2010).

Effective and functioning surveillance calls for a well-equipped lab, sufficient diagnostic resources, qualified personnel, and funds. The four methods of surveillance listed below can be used to combat zoonoses (Chomel, 2008).

- Pathogen detection and identification through pathogen surveillance.
- Using immunological monitoring, serological tracking can find the presence of infections in the RBCs of people or other animals.
- Using evaluation of data based on symptoms, syndrome detection can identify illnesses that are more likely to occur.
- Risk monitoring to find the risk factors behind disease spread.

Every disease management strategy needs a substantial financial investment, which is typically not available to developing nations. For the developing nations to successfully regulate zoonoses, the developed nations and foreign donors must aid, foreign donors agencies include, (WHO), (FAO), (OIE), (USAID), (USDA), (EU), (DFID), (BBSRC), and (DANIDA) could be approached for funding (Gibbs, 2014).

2.6 Host Range of Influenza Viruses

Various species of hosts might be involved in the influenza virus infections cycle based on the genus shown in **Figure 1.**(Skelton & Huber, 2022) Influenza virus are known as "species jumpers" because of their quick transitions between different species of host and their widest host range (Kuchipudi & Nissly, 2018).

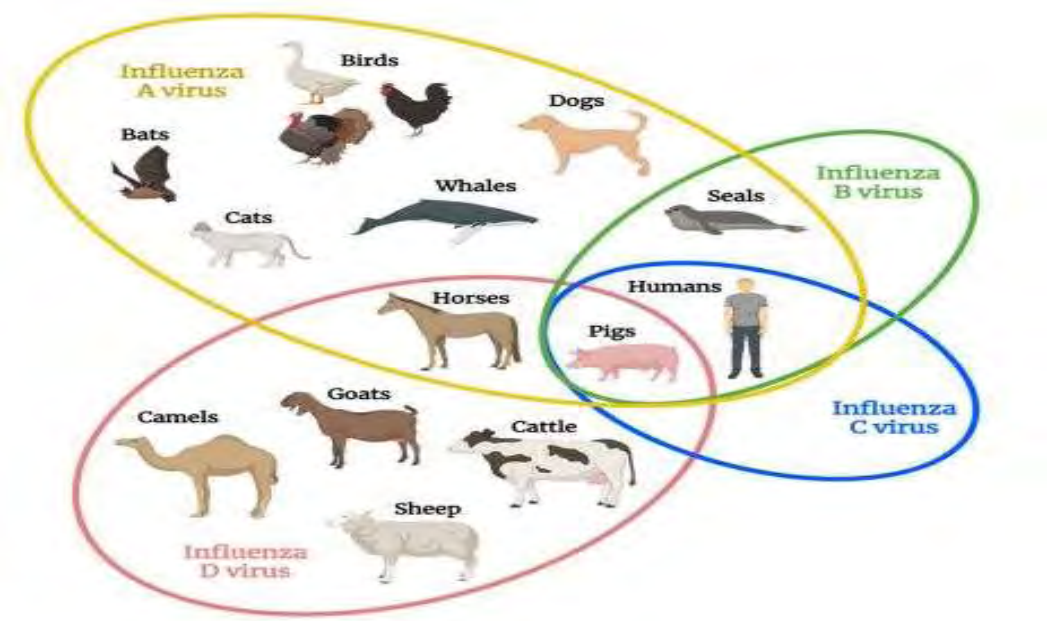


Figure 1 : Influenza virus types and its native hosts.The main host species that each kind of influenza virus naturally affects are listed, with considerable overlap between varieties of influenza virus. Notably, swine are the sole animal known to contract all (Skelton & Huber, 2022).

2.6.1 Wild Birds

Avian influenza and their all serotypes are found in the environmental wild birds. At least one hundred species across 12 of the 50 bird orders have been documented to carry AIV (Stallknecht, Brown, & Swayne, 2008). The most prevalent AIV sources are aquatic and shorebirds of the order *Charadriiformes* (including gulls, terns) and *Anseriformes* (including Mallard ducks), respectively (Stallknecht & Brown, 2016). The main idea is Avian influenza multiplication in these wild bird species occurs mostly in the gastrointestinal tract and that viruses escape in the faecal matter, where they can survive for weeks in water or wet faeces and continue to be contagious (R. G. Webster, Yakhno, Hinshaw, Bean, & Murti, 1978). Avian influenza typically has little lethality in wild birds. However, the introduction of the extremely high lethality

Avian influenza subtype H5Nx into native bird populations is a major killer (Pohlmann et al., 2022).

Furthermore, migratory birds may transmit the virus great distances, it can become enzootic in permanent bird populations, it displays high pathogenicity in domesticated birds, it has infectious potential, and it could infect a variety of mammal species globally (Ramey et al., 2022).

2.6.2 Domestic Birds

The raising of poultry is a significant source of protein for meats. Its dramatic rise in recent decades has resulted in a shift in the spontaneous epidemiology of AIV. All domesticated and indoor bird species, including chickens, turkeys, waterfowl, ostriches, pigeons, quails, gamebirds, and zoo birds, have been found to carry AIVs (Alexander, 2000). While H12 to H16 variant viruses are less common or have not yet been retrieved from poultry (apart from one historical H13N2 variant virus obtained from turkeys), viral infectious agents of the H1 to H11 variant are more constantly identified (Alexander, 2000). In some nations, certain AIV strains are prevalent in poultry, including as H5Nx throughout Asia and Africa, H6N2 in Southern Africa, Korea, and China, H7N3 in Mexico, H7N9 in China, and H9N2 globally (Bi et al., 2020; Xu et al., 2023). The main routes for the initial transmission of AIV into livestock are through close contact with exotic birds or polluted fomites (Nair, 2020).

Additionally, distinguished high lethality AIV infections, primarily H5N1, cause varied reactions in different duck populations (Pantin-Jackwood, Swayne, Smith, & Shepherd, 2013). Main AIV source is thought to be mallard ducks and typically, they are tolerant to the mortality and morbidity caused by AIV. But some H5N1 and H5N8 strains are also extremely pathogenic in mallards (Tang et al., 2009). Therefore, Numerous countries with developing economies have employed vaccination to reduce the losses brought on by low and heigh pathogenicity AIV (Swayne, 2012).

2.7 Mammals

There have been numerous accounts of mammals infected with AIV some of which are discuses.

2.7.1 Humans

Variants N1, N2, H1, H2 and H3, which differ from swine influenza both genetically and antigenically, are the ones that result in seasonal influenza in individuals (Krammer et al., 2018). The three most common subtypes of influenza which affecting humans are H1N2, H1N1 and H3N2 while H1N2 variant is a swine and human influenza A virus re-assortant of the H3N2 and H1N1 viruses that are currently circulating (Dunning, Thwaites, & Openshaw, 2020).

Moreover, The primary mode of propagation is from direct face-to-face exposure via the respiratory system when it comes to human beings, the consequences of cases of influenza can range from minimal to significant, affecting both the lower and upper respiratory tracts (Krammer et al., 2018).

2.7.2 Pigs

Pigs around the world are infected with swine IAV of the three distinct types H1N2, H1N1 and H3N2, which has a significant negative economic impact on pig farming (Bourret, 2018; Hennig et al., 2022). Pig-to-pig infection typically takes place via close proximity and could also happen as a result of polluted things being moved between infected and healthy pigs. Typically, virus replication is limited to the epithelial tissues of the whole respiratory tract, particularly the trachea, lungs, and tonsils while Low fatality rates are caused by the virus, and recovery takes place typically in 7–10 days (Abdelwhab & Mettenleiter, 2023).

Furthermore, controlling (IAV) in pigs often involves vaccination and the concept of biosecurity strategies. Reassortants, which combine genomes elements from human, swine, and avian viruses, make up the majority of swIAV (Waters et al., 2021). around 700 confirmed instances were observed globally between 2010 and 2021, with the majority affecting young people or patients with impaired immune systems (Hennig et al., 2022).

2.7.3 Ferrets

The preferred model for examining the contagious prospective infection, distribution, pathophysiology, and vaccination effectiveness of influenza virus is ferrets. Pig H3N2,H1N1, pdmH1N1, and avian H5N1 Avian influenza virus have all been propagated naturally to pet and colony ferret (Lin, Wang, Wu, Chi, & Wang, 2014; Patterson, Cooper, Yoon, Janke, & Gauger, 2009). Numerous in vivo investigations

have demonstrated that ferrets produce reassortant viruses following co-infections with two separate hIAVs (Richard, Herfst, Tao, Jacobs, & Lowen, 2018).

2.7.4 Seals

Although it may be hard to comprehend, seals do interact with people on a regular basis in places like zoos, parks, and rehabilitation facilities. It has been documented that seals frequently contract individuals seasonal and widespread influenza viruses (Runstadler & Puryear, 2020) It's crucial to note that many AIV infections, such as H10N7, H4N5 and H10N4 have caused mortality and morbidity in seal (Berhane et al., 2022; Bodewes et al., 2016).

Additionally, In the US state of Massachusetts, in 1979, there was an instance of seal-H7N7 spreading to people who developed conjunctive inflammation (R. Webster, Geraci, Petursson, & Skirnisson, 1981).

2.7.5 Dogs

The worldwide total number of domestic dogs is predicted to be over 900 million, even though it is hard to establish an accurate assessment of the overall dog population (Wasik, Voorhees, & Parrish, 2021). Dogs kept as pets have a close relationship with people. In addition to the recognised origins of canines H3N2 and H3N8 influenza viruses, several human seasonal and pandemic H1N1, H3N2, as well as a variety of AIVs (such as H9N2, H5N2, H10N8 and H6N1) have been isolated in dogs in recent years (Klivleyeva, Glebova, Shamenova, & Saktaganov, 2022; Lin, Wang, Chueh, Su, & Wang, 2015).

Interestingly, A person was infected in China in 2022 by an AIV subtype H3N8. An apparently fit dog living in the patient home had viral RNA found in its nasal sample (Bao et al., 2022).

2.7.6 Cats

Cats are among the most popular pets and frequently interact with people. In contrast to dogs, occasional reports of IAVs in cats have been reported (Wasik et al., 2021). Cats are particularly vulnerable to a variety of IAVs, such as the human seasonal and epidemic influenza infections as well the varying serotype of avian influenza virus H9N2, H5N6, H5N1 and H7N2 (Zhao et al., 2020). A veterinary professional and an

employee of an animal shelter in the United States were both reported to have contracted AIV H7N2 from cats in 2016 (Belser et al., 2017).

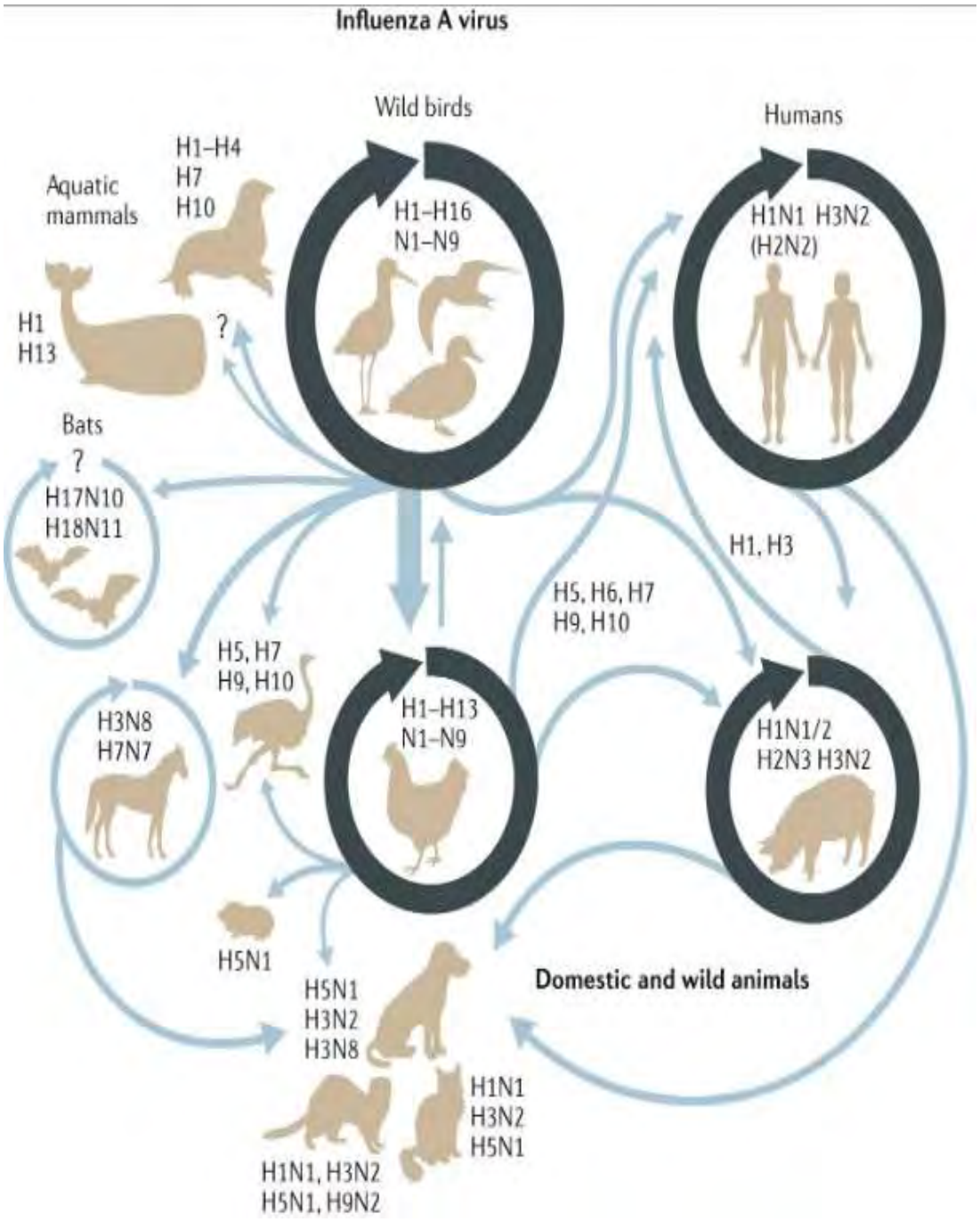


Figure 2 : Influenza A viruses (HA variants 1-16) proliferate in the naturally occurring wild and domestic bird pool.variants from this pool can migrate into a variety of species, sometimes through secondary hosts and additionally through adaptive variations (J. S. Long, B. Mistry, S. M. Haslam, & W. S. Barclay, 2019).

2.8 Bats Associated Influenza Virus

The second-most varied group of mammals, bats are found on every continent except Antarctica and have been implicated in the spontaneous transmission of several zoonotic virus infections (S. Rahman, Ullah, Shinwari, & Ali, 2023). Approximately 20% of all mammal species are bats, which are taxonomically diversified. They are the primary nighttime aerial predators and the sole mammal with the ability to fly with propulsion (D. T. Hayman, 2016). Classical classification of bats into two suborders included *Megachiroptera* (megabats) and *Microchiroptera* (microbats) both of which included the *Pteropodidae* family of Old-World fruit-eating bats these members tend to be bigger, fruit-eating bats lacking echolocation. They rely instead on vision and smells (D. T. Hayman, 2016; Teeling et al., 2005). The *Phyllostomidaefamily* includes new world bats. One of the most biologically diversified bat families, this group has developed species that can eat practically every food source, including fruits, pollen, nectar, pests, and vertebrates (D. T. Hayman, 2016; Kunz, 2013). It has been hypothesized that the variety of bats is a method for promoting the variety of the viruses they host (Calisher, Childs, Field, Holmes, & Schountz, 2006; Luis et al., 2013). Several animal-borne viruses have been identified in the past ten years in a variety of bat species from across the globe, including those from Europe, the Americas (North and South), Asia, Africa, and Oceania (Zhou et al., 2021). More than 200 distinct viruses have so far been found in or extracted from bats. These bat-borne viruses pose a zoonotic and epizootic concern because some of them have led to animal and human diseases, including *rabies, Ebola, MERS,SARS and IAVs* (Maher & DeStefano, 2004). The degree of individual preadaptation and the virus‘s capacity to overcome host limitations strongly influence the risk of zoonotic spread and the accompanying outbreaks that arise from an influenza virus. Avian influenza A virus, on the other hand, encode segments of genes with specific to a species markers that enable effective replication in avian cells, though potentially in human cells (Jason S Long, Bhakti Mistry, Stuart M Haslam, & Wendy S Barclay, 2019). The ability of New World bat IAVs to employ the individual's MHC-II homologue human leukocyte antigen-DR isoform (HLA-DR) for cell entrance further suggests that they may be capable of infecting humans (Karakus et al., 2019).

Moreover, Numerous infectious viruses naturally exist in bat species as reservoirs. Recently, two unique H17N10 and H18N11 IAV were found in the a fruit-eating bats

(*Artibeus planirostris*) and yellow-shouldered bats (*Sturnira lilium*) individually, in Guatemala and Bolivia (Ciminski & Schwemmle, 2019). Additionally, H9N2 viruses that are significantly connected to AIV H9N2 have been found Egyptian fruit bats (*Rousettus aegyptiacus*) (Kandeil et al., 2019). The Avian associated bats H9N2 showed the capacity to bind to 2,3- salicylic acid receptor at higher levels than 2,6-salicylic acid receptor, in contrary to H17N10 and H18N11, which recognize MHCII as a receptor (Kandeil et al., 2019). However, inoculation of cell lines from mammals and models of animals with H18N11 virus demonstrated that this virus can pick up mammal-adapting alterations that might boost its zoonotic capability (G. Zhong et al., 2020).

2.9 Transmission of Influenza Virus

It is beyond a doubt that influenza A viruses stand out for their repeated transmission to a variety of host species and extremely quick host adaptability (Johnson & Hariharan, 2017). Most viral influenza infections are host specific. Mammals rarely encounter avian viruses that cause influenza spontaneously. Although various avian species can respond differently to the same virus that causes influenza. However, interspecies leaping happens regularly (Cheng, To, Tse, Hung, & Yuen, 2012).

Furthermore, Humans were previously known to contract the H5N1, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N7 influenza viruses that originate directly from bird species and poultry (Chen et al., 2013; To et al., 2012). According to evolutionary and epidemiology study, numerous viruses associated with influenza have also been transmitted directly across numerous types of mammals, birds, and nonhuman mammals (Amonsin et al., 2006).

2.10 General Features of Influenza Virus Infections

Influenza is a serious respiratory condition that can range in severity from moderate to severe. The *Orthomyxoviridae family* contains the pathogenic viruses that cause these illnesses (Taubenberger & Morens, 2008), are four different influenza virus genera AlphaInfluenzavirus, BetaInfluenzavirus, GammaInfluenzavirus and DeltaInfluenzavirus belonging to this family and each is made up of a single species include, influenza A, influenza B, influenza C, and influenza D individually (Hutchinson & Yamauchi, 2018).

2.11 Various Types of Influenza Virus

2.11.1 Type A Influenza Virus (IAV)

Influenza A virus infections continue to remain a major cause of morbidity and mortality globally and therefore represent major infectious agents from a medical perspective. Probably the most devastating consequences of influenza in the past century was the 1918 epidemic of Spanish flu, which killed over forty million people (Peter Palese, 2004), a disease that affects humans, certain mammals, and bird and causing the flu. Sub types of this RNA virus were discovered in wild bird specimens. On rare occasions, it can spread from wild to domesticated birds, which can result in serious illness, incidents, or influenza-related human global epidemics (Organization, 2008).

Influenza According to predictions from the WHO, viruses associated with influenza A (IAVs) spread throughout the world and cause an estimated 1 billion diseases, 3-5 million incidences of serious disease, and 3-5 lac deaths. The impact of the global pandemic of influenza is determined by a number of variables like the viral load of the pandemic virus variant and the community's degree of previous immunity (Tong et al., 2012).

2.11.2 Structure and Genetics of (IAV)

Influenza A is an RNA virus with a single-stranded, segmented viral genome and a negative sense. Hemagglutinin type (H number) and neuraminidase type (N number) are used to identify the different sub types (Control, 2013). In terms of organization, influenza types A, B, C, and D are highly identical to each other (Nakatsu et al., 2018). The viral particulate, also known as the virion, is oval in appearance and ranges in size from 80 to 120nm (Noda, 2012) Due to influenza's polymorphic nature, each particle's lengths vary greatly and can exceed several tens of micrometers, resulting in filamentous virions (Dadonaite, Vijayakrishnan, Fodor, Bhella, & Hutchinson, 2016). The viral genomes of all influenza type A viruses share a common makeup despite their diverse forms.

Furthermore, based on its composition properties (HA) and (NA) are the two significant peptides that may be seen on the outermost layer of viruses. HA is a molecule that facilitates the entrance of the genome of the virus into the intended cell as well as the attachment of the viral particle to target tissues while a lot of the

numerous unproductive areas of attachment in mucous are released by NA (Cohen et al., 2013).

The shape and structure of flu virus molecules is influenced by a few viral protein components, including HA, NA, M1, and M2. This morphology is a hereditary feature (Burleigh, Calder, Skehel, & Steinhauer, 2005; Roberts, Lamb, & Compans, 1998). In the lipid component of the viral envelope exist three viral protein complexes called HA, NA, and M2 (Lamb & Takeda, 2001). The short chains of water-resistant amino acids that make up HA and NA, two spikes glycoprotein, bind them in the phospholipid barrier. To attach receptors and fuse membranes, the homotrimer HA is required While NA is a homotetramer whose purpose is to expel the viral offspring and breaking down molecules of sialic acid from forming glycoproteins to damage receptor. M2 peptide is an essential transmembrane homotetramer that acts as a type of ion channel following viral replication to acidify the inside of the viral particulate (Pinto, Holsinger, & Lamb, 1992). Additionally, there is an M1 peptide structure beneath the viral phospholipid membrane (Ruigrok, Calder, & Wharton, 1989).

Furthermore, Each of the 8 vRNA regions are joined to the NP and the viral genome RNA polymerases within the virion to create ribonucleoprotein (RNP) (Lamb & Choppin, 1983). It is believed that NP, being the most prevalent peptide in the virion, interacts via the phosphate-sugar base of the vRNA pattern (Baudin, Bach, Cusack, & Ruigrok, 1994), binds with roughly 20 vRNA sequences (Lamb & Takeda, 2001).

Three different polymerase subunits PB2, PB1, and PA combine to form the RNA polymerase complexes (Hsu, Parvin, Gupta, Krystal, & Palese, 1987). Indicating that inside the viral particulate, the RNA polymerase engages in interaction with each end of the Vrna (Klumpp, Ruigrok, & Baudin, 1997) In small concentrations, the NS2 peptide is also found in virions (Richardson & Akkina, 1991) is shown in **figure 3.**

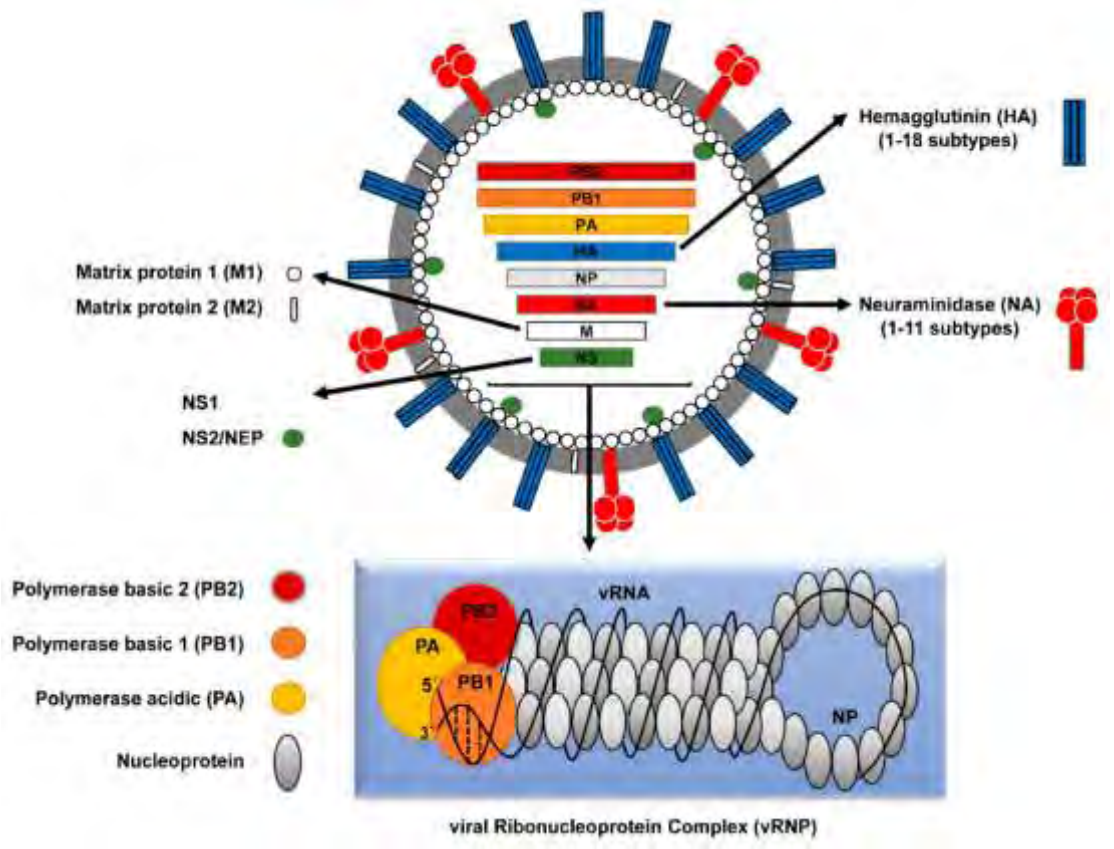


Figure 3 : Structure and genetics of IAV (Richardson & Akkina, 1991).

2.11.3 Genomic Organization of the Influenza A Virus

The full influenza A virus's 13,588 base length genomes is split into 8 RNA sections, each of which codes for a maximum of 10 but possibly as many as 14 peptides, according on the variant (Eisfeld, Neumann, & Kawaoka, 2015). Genomic mRNA of (IAV) virus from region 1 and 3 to 6 are mostly monocistronic. While Certain isolated viruses have an additional open-reading region in their region 2 viral mRNA. However, mRNA from region 7 and 8 having different splicing processes to produce proteins (Lamb & Takeda, 2001). The lengths of the RNA from the virus fragments and the protein that are expressed are listed in Table 3. Especially the NS1 peptide from region 8 and the PB1/F2 peptide from region 2 are non-structural peptides out of the entire group (Cheung & Poon, 2007).

Table 3 : The influenza virus RNA and the correspondingly encoded proteins.

vRNA segments	vRNA Length (bps)	Encoded Proteins
Segments-1	2341	PB2
Segments-2	2341	PB1 and PB1-F2
Segments-3	2233	PA
Segments-4	1778	HA
Segments-5	1565	NP
Segments-6	1413	NA
Segments-7	1027	M1 and M2
Segments-8	890	NS1 and NS2(NEP)

2.11.4 First segment of viral RNA (PB2) protein

first region of the influenza A virus (IAV) PB2 is a component of the influenza viral processor that is encoded by a virus (Perales & Ortín, 1997). The minimal number of peptides necessary for viral replication and transcription is generally acknowledged to consist of Basic polymerase-2 protein, basic polymerase-1 protein, polymerase Acidic and Nucleoprotein (Honda, Mizumoto, & Ishihama, 2002). A nuclear localization signature found in Basic polymerase-2 (PB2) (Mukaigawa & Nayak, 1991) allows it to be delivered into infect tissues nuclei where it is used for viral expression and replications (I. M. Jones, Reay, & Philpott, 1986). Furthermore, the creation of the capped structures for viral messenger RNA depends on the basic protein PB2. The cap binding region is most likely close to the carboxy terminus, according to this protein (Masunaga, Mizumoto, Kato, Ishihama, & Toyoda, 1999) PB2 contains endonuclease action and produces cap primer for infectious mRNA production by using host mRNA (Shi, Summers, Peng, & Galarza, 1995).

In addition to the physiological duties listed above, PB2 may have a significant role in influencing the infectiousness of influenza A virus (IAV) (Hatta, Gao, Halfmann, & Kawaoka, 2001).

2.11.5 Second segment of viral RNA (PB1) protein

The Second region of viral RNA encodes the RNA polymerase component PB1. Multiple sources of scientific research claimed that PB1 is an RNA processor itself(Asano & Ishihama, 1997). The 4 RNAs-dependent enzymes recognized patterns

are found in PB1(Poch, Sauvaget, Delarue, & Tordo, 1989), and the enzyme polymerase activities was inhibited when these patterns were mutated (Biswas & Nayak, 1994). The essential domain of PB1 that interact with the various other host enzymes subunit have been discussed in several publications.

Moreover, research on the influenza viral RNA enzymes immunoassay revealed that PB1 has separate binding areas for PA and PB (Digard, Blok, & Inglis, 1989).

2.11.6 Third segment of viral RNA acidic polymerase (PA) protein

The third region of viral RNA encodes the PA proteins, which is the least component of the influenza RNA polymerase chain. It features nuclear localization communications, like the other influenza virus enzymes subunits necessary for transportation into the cell's nucleus (Nieto, de la Luna, Bárcena, Portela, & Ortin, 1994). this protein required for viral transcription and replication (T. Huang, Palese, & Krystal, 1990).

Furthermore, it‘s important to note that PA causes proteolysis processes in cells with infection (Sanz-Ezquerro, De La Luna, Ortín, & Nieto, 1995). However, this characteristic is unrelated to any recognized viral activity, and it is still unclear how important this information are (Sanz-Ezquerro et al., 1995).

2.11.7 Fourth segment of viral RNA hemagglutinin (HA) protein

The fourth region of viral RNA encode hemagglutinin protein. these peptides unite as homotrimers in viruses controls the attachment of viral parts to the cell's exterior chemical sialic acid receptor in host (Nicholson, Webster, & Hay, 1998). Additionally, it serves as the main site for immune system antibodies that neutralize substances (Staudt & Gerhard, 1983). As a result of a polypeptide progenitor called HA0, Hemagglutinin is created. After translational process This polypeptide progenitor splits into the Hemagglutinin 1 and Hemagglutinin 2 subunits. Additionally, this splitting permits the native Hemagglutinin protein to experience a structural shift, which is caused by acidic conditions and is necessary for cell fusion over a protective barrier (Skehel et al., 1982).

2.11.8 Fifth segment of viral RNA nucleoprotein (NP)

fifth Region of viral RNA carries the Nucleoprotein encoding. It‘s a basic peptide that has been phosphorylated after and its overall charge is positive at pH zero (Kistner,

Müller, & Scholtissek, 1989; Winter & Fields, 1981), crucial elements for multiplication and transcription (T. Huang et al., 1990). RNA-binding motif can be found at the end of the amino chain of nucleoprotein. Therefore, it has been proposed this Nucleoprotein encapsidates the RNA from the virus in a sequence-neutral manner (Albo, Valencia, & Portela, 1995).

Moreover, viral RNA nuclear translocation has been additionally found to depend on the NP protein (Martin & Heleniust, 1991). It is a transport peptide and has nuclear localization signal (Neumann, Castrucci, & Kawaoka, 1997). It is thought that NP mediates the transfer of incoming viral RNPs from the viral element into the cell's nucleus over the initial stages of the infection (O'Neill, Jaskunas, Blobel, Palese, & Moroianu, 1995). While future generations viral RNAs linked to NP, M1, and NS2 are transported to the cell's cytoplasm for viral assembly during the late phase of infections (Elton et al., 2001).

2.11.9 Sixth segment of viral RNA neuraminidase (NA) protein

The sixth region of viral RNA encodes NA peptides. The 3-D structure NA monomers is made up of four domains, including a cytosolic domain, a domain located inside the membrane, a slender stalk, and a box-shaped conical head (Varghese & Colman, 1991). The NA is an exterior glycoprotein, and its glycosylation may play a significant (though not exclusive) role in the neurological infection of influenza viruses (S. Li, Schulman, Itamura, & Palese, 1993).

Furthermore, the role of neuraminidase function throughout the influenza virus development process remains unknown (C. Liu, Eichelberger, Compans, & Air, 1995). When NA activity is suppressed, offspring virus particles adhere to one another or the cell interface to form huge complexes (P Palese & Compans, 1976). New evidence suggests that the unique cytosolic tail of NA regulates virion shape and pathogenicity (Bilsel, Castrucci, & Kawaoka, 1993)

2.11.10 Seventh segment of viral RNA matrix proteins (MP)

The seventh region of viral RNA encode two types of proteins M1 and M2, both of proteins are the product of this region, M1 as a Colinear transcribe result while M2 as a result from spliced mRNA (Shih, Nemeroff, & Krug, 1995).

The matrix M1 protein creates a layer within the virus particle in order to isolate the protein known as RNP from the viral cell membrane (Ruigrok et al., 2000). and it participates in the synthesis and deconstruction of (IAV)by interacting with the paired viral RNA and peptide elements of RNP (Ye, Liu, Offringa, McInnis, & Levandowski, 1999). Multiple roles for the virus are said to be played by the M1 protein. First, it stops transcription of viruses by binding to RNA in a non-specific way (Watanabe, Handa, Mizumoto, & Nagata, 1996). It also carries signals for nuclear localization and appears to control viral (vRNP) nuclear transport. Several investigations have shown that (vRNP) nuclear export is linked to the matrix protein (X. Huang, Liu, Muller, Levandowski, & Ye, 2001). According to prior research, the interaction between M1 protein and vRNP is necessary for the nuclear export of vRNP because when M1 protein is synthesised at high temperatures (41oC), the entry of vRNP into the cell's cytoplasm is prevented (Sakaguchi, Hirayama, Hiraki, Ishida, & Kim, 2003). M1 protein also attaches to the cell's outer membrane and appears to have an impact on viral construction, budding, and viral shape (T. Liu, Muller, & Ye, 2002). According to recent speculation, the matrix protein may be able to bind to and limit the amidolytic action of the virus's RNA polymerase PA component (Hara et al., 2003).

Additionally, the matrix M2 peptides, a disulfide-bonded homotetramer, is an essential part of the membrane's composition (Holsinger & Alams, 1991).while ion channel activity is present in the M2 tetramer for PH regulation (Ciampor, Thompson, Grambas, & Hay, 1992). The ion channel operation of M2 permits the inside of the arriving viral particulate to be acidified in the endosome of cells with infection Because it enables incoming vRNPs to separate from M1 peptides for nuclear import, acidifying of the viral particulate is thought to be crucial for viral propagation or replication (Martin & Heleniust, 1991) However, it has also been observed that M2 ion channels action helps keep the pH level in the Golgi bodies vesicle which helps to stabilize the natural geometry of freshly synthesized HA during intracellular movement for viral replication (Takeuchi & Lamb, 1994). Recent research suggested that the M1 protein matrix would be the only factor controlling the filamentous morphology of influenza A virus (Elleman & Barclay, 2004).

2.11.11 Eighth segment of viral RNA non-structural proteins (NS)

The eighth region of viral RNA encodes two types of proteins NS1 and NS2, NS1 as a colinear transcribe result while NS2 as a result from spliced mRNA (Alonso-Caplen & Krug, 1991).

The single nonstructural peptide of the influenza virus is called NS1. It mostly builds up in the cell nucleus and is an oligomer (Greenspan, Palese, & Krystal, 1988). The NS1 peptide appears to control the production of virus and cellular protein via interacting with various molecules of RNA include, cellular RNA poly(A) tail, viral ribonucleoprotein (VRNP), viral RNA, dsRNA and snRNA (Qiu & Krug, 1994; Qiu, Nemeroff, & Krug, 1995) by promoting the translation of infectious mRNA, NS1 protein also seems to increase the synthesis of viral proteins (De La Luna, Fortes, Beloso, & Ortín, 1995).

Moreover, in earlier research, the NS2 protein was thought to be a nonstructural peptide. But additional research has revealed that NS2 is only minimally integrated into viral fragments (Richardson & Akkina, 1991). There is also an matrix protein-1 binding motif in the carboxyl-terminal portion of NS2 which regulate and control the physiology of M1 protein (Ward et al., 1995). It has been suggested to rename the NS2 peptide NEP (viral nuclear export proteins) based on the findings that it carries a nuclear export signals and aids the export of viral RNP (O'Neill, Talon, & Palese, 1998).

CHAPTER 3

MATERIALS AND METHODS

3 Materials and Methods

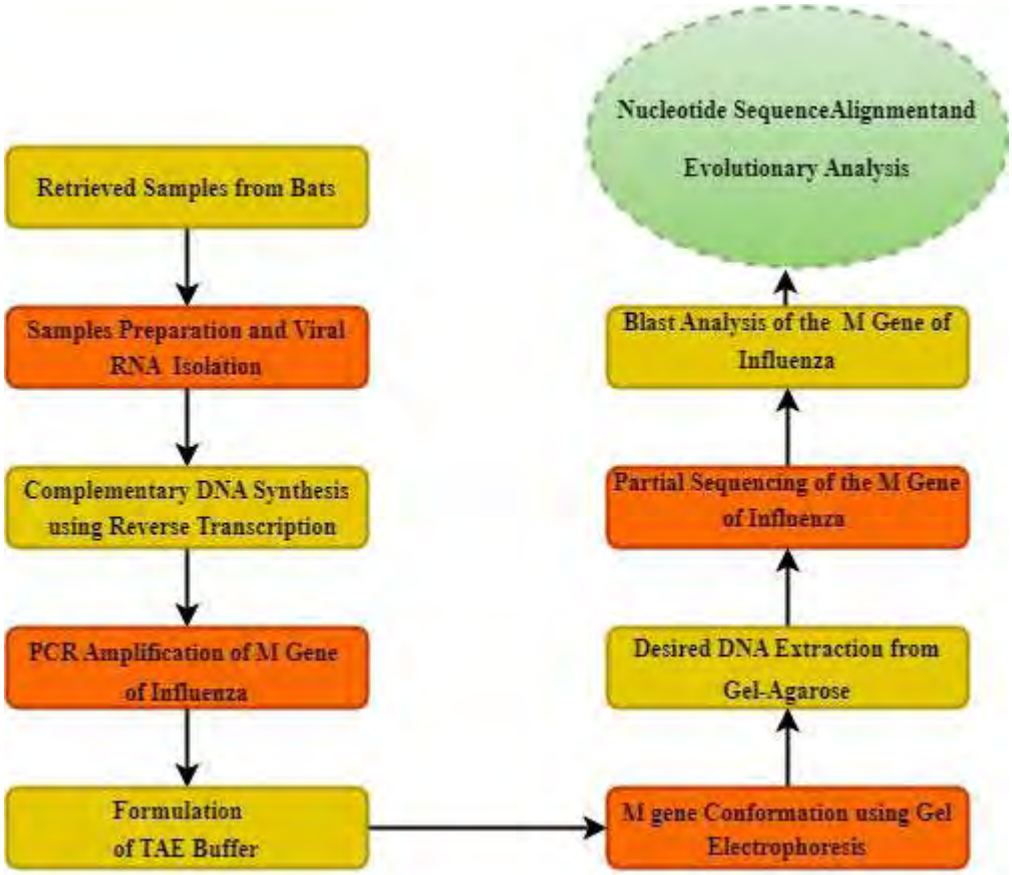


Figure 4 : The entire procedure of the molecular detection of bats associated with Influenza virus is shown in schematic representation.

3.1 Samples collection from bats

The sum of 50 specimens of bat guano were taken from the natural surroundings in various parts of district Swat province Khyber Pakhtunkhwa. The habitats of the tested bats comprised trees, occupied buildings, caverns, and residences, all of which had significant levels of inadvertent bat-human engagement. Hygienic sheets of plastic were placed beneath the identified bat roosting locations for the purpose of collecting samples primarily in the morning and evening time, and specimens were taken up right away, maintained in 500µL VTM (viral transport medium in eppendorf tubes) and transported to the Infectious Diseases and Molecular Pathology Lab of Department of Biotechnology Quaid-i-Azam University and held there at -20°C prior additional processing.

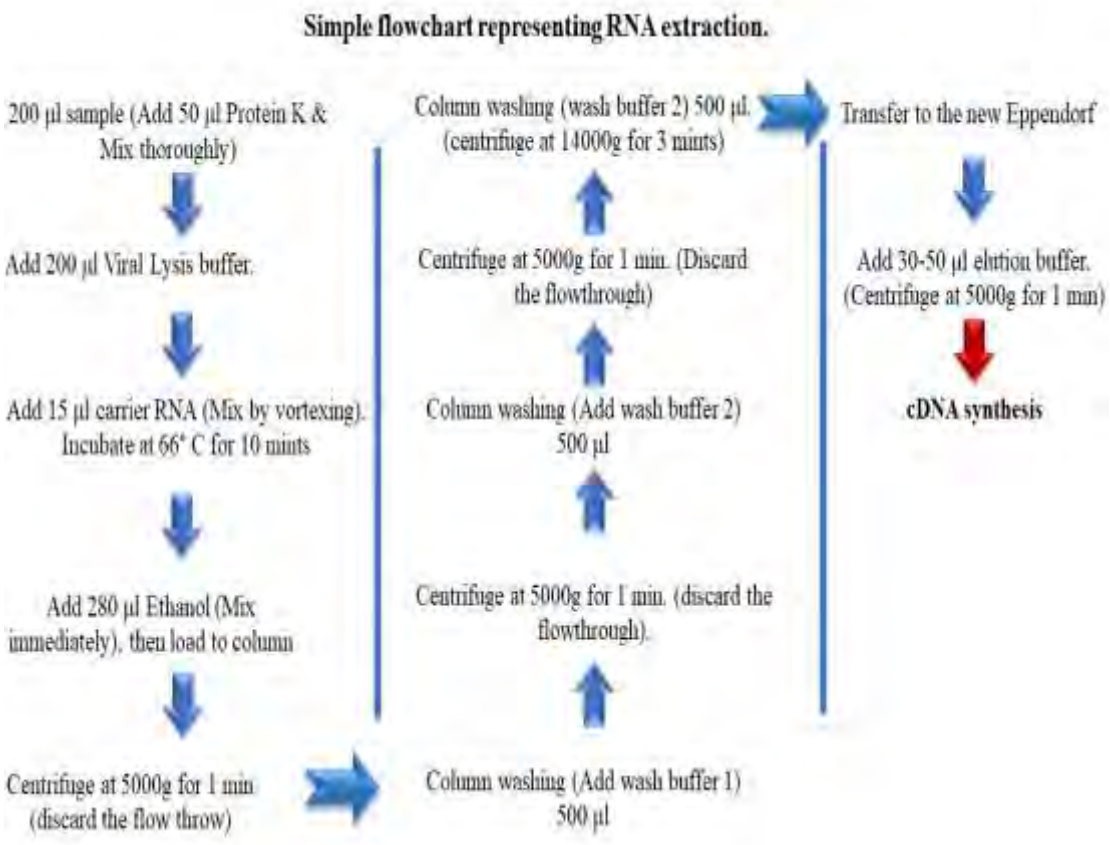
3.2 Inclusion and exclusion criteria

Influenza positive samples confirmed by PCR were included. While those negative samples were excluded from the study.

3.3 Samples preparation and viral RNA isolation

The entire investigation was carried out in a biosafety cabinet class II (BCL- II) while taking all necessary safety measures. The manufacturer's recommended procedure was followed while extracting influenzas RNA using a viral RNA extraction kit (GF-1 Viral Nucleic Acid Extraction Kit, Vivantis Technologies, Malaysia). After being vortexed for 1 minute to homogenise the specimens obtained, then underwent centrifuge treatment at 13000 rpm for 3 minutes to obtain clear supernatant.

The first step was labelling autoclaved microfuge tubes with precise lab values. Following the use of 50µl of enzyme proteinase K, which began the process of digest protein and remove contamination from the specimen, the 200µl tagged microfuge tubes of serum samples were included and the material was then carefully mixed. 200µl of viral lysis buffer solution were poured from the viral RNA isolation kit, 15µl of carrier RNA were placed into the tube including the sample, and the mixture was vortex-mixed for 10–15 seconds. After incubating the sample combination for 10 minutes at 66°C in the heated block, 280µl of 100% ethanol was poured into each tube and homogenized quickly to avoid ethanol precipitation because of the amount present. The specimen mixture was then put into the kit's supplied columns and centrifuged at 5000g for one minute, the flowthrough was removed after the centrifugation process and the column itself was sterilized with 500µl of washing buffer 1 and agitated once more for 1 minute at 5000g. 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 filter column was spun up at 14000 g for 3 minutes after being cleaned with 500µl of washing buffer, the flowthrough was then removed. After transferring the filter column into a fresh microfuge tube, and adding 30–50µl of elution buffer, and centrifuged for 1 minute at 5000g, the RNA of the virus was kept at –20 °C.



3.4 Complementary DNA synthesis using reverse transcription

Complementary DNA (cDNA) were synthesized using the extracted RNA as a template. First strand cDNA Synthesis Kit (RevertAid, ThermoScientific) was used. The subsequent materials were employed in a total of 40µl of reverse transcription reaction: 16µl of isolated templat RNA along with 4µl universal primers Uni-13 (TCA TCTTTGTTCC) and Uni-12 (AGCAAAAGCAGG), 5.5µl PCR water, 4µl dNTPs (10 mM), 8µl 5x reaction buffer, 2µl RevertAid RT enzyme and 0.5µl RiboLock (RNase Inhibitor) were mixed gently, and total reaction volume adjusted to 40µl as shown in Table 4. All the components of reaction mixture were mixed and incubated for 5min at 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 5.

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

Serial No.	Reagents	Quantity
1	5x reaction buffer	8µl
2	Uni-12(AGCAAAAGCAGG), or Uni- 13(TCATCTTTGTTCC) primers	4µl
3	10 mM dNTP Mix	4µl
4	Template RNA	16µl
5	ddH2O	5.5µl
6	RiboLock (20U/µL)	0.5µl
7	RevertAid RT enzyme (200 U/µL)	2µl
	Total volume	40µl

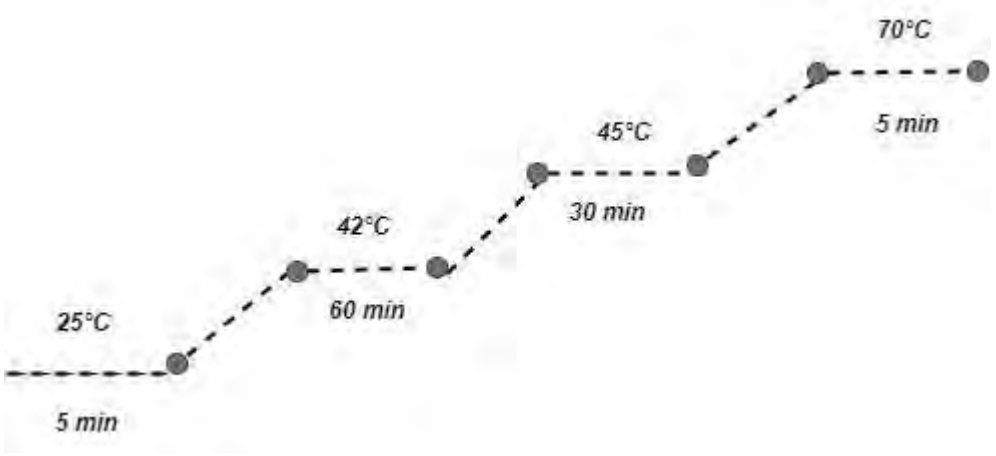


Figure 5 : Representation of optimized conditions for cDNA synthesis.

3.5 PCR amplification of the M gene of influenza

M-229L (AAGCGTCTACGCTGCAGTCC) and M-229U (TTCTAACCGAGGTCGA AAC) were used as the forward and reverse primers respectively in a nested polymerase-chain reactions for the M gene of influenza virus recommended by Bi and colleagues to determine if the specimens was influenza negative or positive (Bi et al., 2016). Created 20µl PCR reaction contain Phusion HF master (Thermo Scientific, USA) mix 10µl, forward and reverse primers 1.5 µl, cDNA as template 6 µl and PCR water 1 µl are given in below Table 5. The cyclic condition for PCR were as follows:

96°C for 5mins following 35 cycles-of 96°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 6.** The reaction was hold at 4°C for infinity.

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

Serial No.	Reagents	Quantity
1	Phusion master mix	10µl
2	Forward primer M-229L (AAGCGTCTACGCTGCAGTCC)	1.5µl
3	Reverse primer M-229U(TTCTAACCGAGGTCGAAAC)	1.5µl
4	Template RNA	6µl
5	PCR water	1µl
	Total volume	20µl

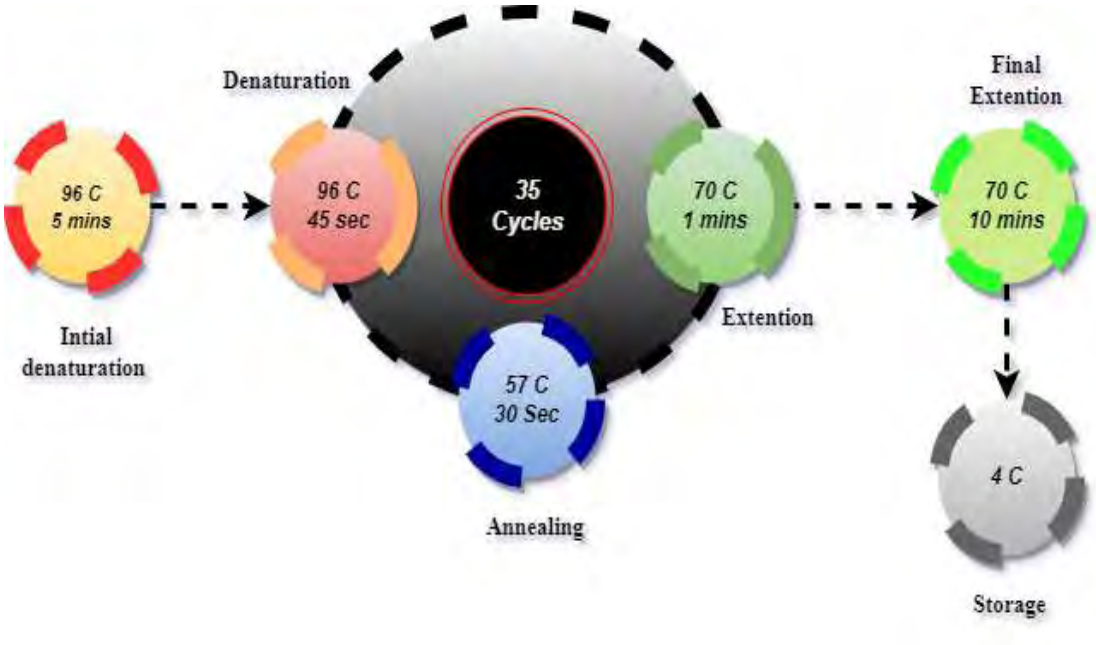


Figure 6 : Polymerase chain reaction's cyclic conditions for M gene partial region.

3.6 Formulation of TAE buffer

In the procedure of gel electrophoresis, the running buffer Tris Acetate EDTA (Ethylenediaminetetraacetic acid), also known as TAE, is used to segregate DNA in agarose gels. To make TAE buffer, reagents including Tris base, glacial acetic acid, EDTA, and double distilled water (ddH2O) were utilised. 48.4g of (Tris base) and 11.4ml of (glacial acetic acid) are needed to create one litre of the 10X (TAE) buffer were combined with 800ml of ddH2O and properly mixed. Then 200ml of ddH2O and 3.7g of EDTA were combined to determine the pH of the EDTA. When EDTA entirely dissolves in water and turns apparent it indicates that the pH of the solution is appropriate. Two mixtures were then combined to create the final 1000ml volume. Then Ten millilitres of the prepared 10X buffer were mixed with 90 millilitres of ddH2O to create 100 millilitres of 1X TAE buffer (the working buffer).

3.7 M gene conformation using gel electrophoresis

For the validation of the synthesized products of PCR were tested on a 1.5% agarose gel. 0.9 g of powdered agarose was liquefied in a 60 ml (1X buffer) solution to make the gel. After heating the conical flask for 30 seconds in the microwave, it was then allowed to cool. After cooling, 2µl of ethidium bromide was added into the 1.5% agarose solution. This mixture was then poured onto a gel casting tray that had already been fitted with the proper comb and allowed to sit until it solidified. Additionally, a running solution of 1X TAE buffer was employed in the gel chamber. A 6X loading dye was mixed with the product of PCR before it was placed into the gel's wells. For a measure of the magnitude of generated products of PCR, a 100 bp DNA ladder (Thermo scientific) was placed in a separate well. After that, the gel was exposed for 30 min at 90V, and then shortly after the gel electrophoresis operation was finished, the gel was examined in an Ultraviolet trans-illuminator to see the amplified DNA.

Table 6 : Ingredients for the gel electrophoresis.

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

3.8 Desired DNA extraction from gel-agarose

Using Gene JET Gel Extraction Kit (Thermo Scientific), the amplified segment was purified after being excised from the gel. With the use of a fine blade, the desired DNA gel piece was removed and placed in a pre-weighed sterilized microfuge tube. Measuring the removed gel was done with a digital weighing balance. Following that, 50µl of binding-buffer solution was placed into the tube in a weight-to-volume ratio of 1:1. 50 mg of gel sliced was mixed with binding buffer, and the combination was heated using a heat block at 60°C for 10 minutes. Then, add 800µl of the soluble gel liquid to the columns, centrifuged for one minute at 12000 rpm. For sequencing reasons, 100µl additional binding solution was added to the columns after the flow through was discarded. A second centrifugation of the mixture at 12000 rpm for 60 seconds was performed before discarding the flow through. The 700µl of Wash Buffer was added, centrifuged for 1 minute at 12000 rpm, and the flow through was discarded. The residual wash buffer was then removed from the empty columns by centrifuging them for 1 minute at 12000 rpm. After that, 35µl of the elution buffer was added to the filter column in a sterilized microfuge tube. This tube was taken out after 1 minute of centrifugation at 12000 rpm, and the purified gene fragment was kept at -20°C. For verification, 5µl of the acquired DNA was run once more on a 1.5% agarose gel and seen through an ultraviolet trans-illuminator.

Figure 7 : Gel purification procedure of amplified PCR products**.**

3.9 Partial sequencing of the M gene of influenza

Sanger sequencing method was used to sequence the purified products of PCR (M gene; matrix protein) with ideal primers (forward and reversed). BioEdit was used to

visualize the sequencing results (chromatograms). The resulting nucleotide sequences were saved in "FASTA" format for further processing. MEGA 11, was used for assessing phylogenetic relationships among the current study sequences (n=5) and reference sequences (n=50).

3.10 Blast analysis of the M gene of influenza

Homology analysis of the query sequences (QAU-HA2, QAU-HA3, QAU-HA4, QAU-HA5, QAU-HA9) was performed using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/) with the known Influenza nucleotides sequences present in NCBI GenBank. Reference sequences (n=50) were obtained from Genbank.

3.11 Nucleotide sequence alignment and evolutionary analysis

Utilizing genetic information resources for the Influenza M genes that are accessible in the National Centre for Biotechnology Information (NCBI), the resulting sequences were examined. BioEdit or MEGA-11 tools was used to align the viral sequences that were inferred (Sudhir Kumar, Stecher, Li, Knyaz, & Tamura, 2018). Phylogenetic tree structures were constructed using the Neighbor-Joining method, and 1000 boots traps in MEGA-11 were used to calculate bootstrap scores.

3.12 Identification and alignment of amino acid chains sequences

Present sequences of nucleotide (n = 5) were converted to amino acid sequences using the Expasy tool, and then aligned with reference sequences for Bats associated influenza Virus using the CLC sequence viewer (version 8) program. MEGA-11 software was also used to align the amino acid sequences.

3.13 Primary and secondary structure of proteins

To predict primary and secondary structure of Influenza M gene samples with its reference sequence, and online self-optimized predication server PSIPRED was used (Dey et al., 2022).

CHAPTER 4

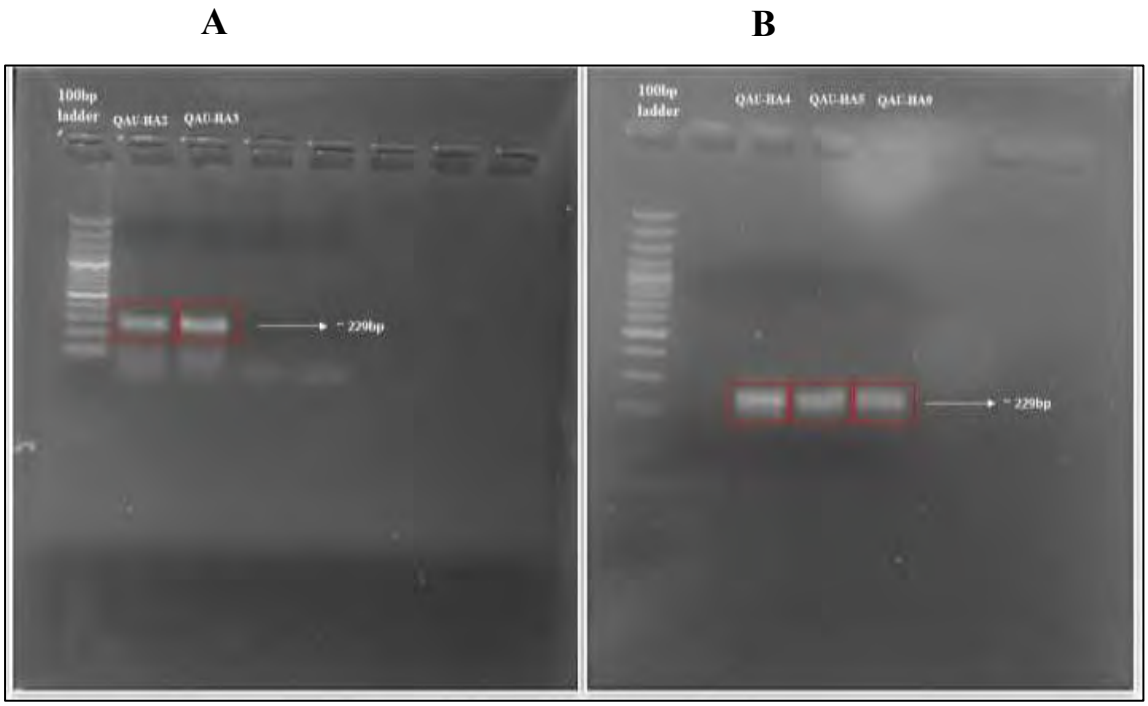
RESULTS

4 Results

In the current study, specimens of bat guano (n = 50) were taken from the various regions of district Swat province Khyber Pakhtunkhwa from 2021 to 2023. Two distinct regions of district Swat were selected for collection of these samples (35 bats specie specimens from tehsil kabal and rest of 15 bats specie specimens from tehsil Babuzai) for the detection of Influenza virus, 8 were identified positive. Tehsil Babuzai was the source of all the positive specimens.

4.1 Detection of bats Influenza virus using M gene

PCR was used to use gene-specific primers (M-229L as forward primer and M-229U as a reverse primer) to amplify these specimens. The amplification was further confirmed by running the amplified product mixed 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. The 15 specimens that were taken from tehsil Kabal were all Influenza-free. The schematic diagram, location map and agarose gel showing the Influenza virus M gene 229 base pairs are both displayed in Figure 8.



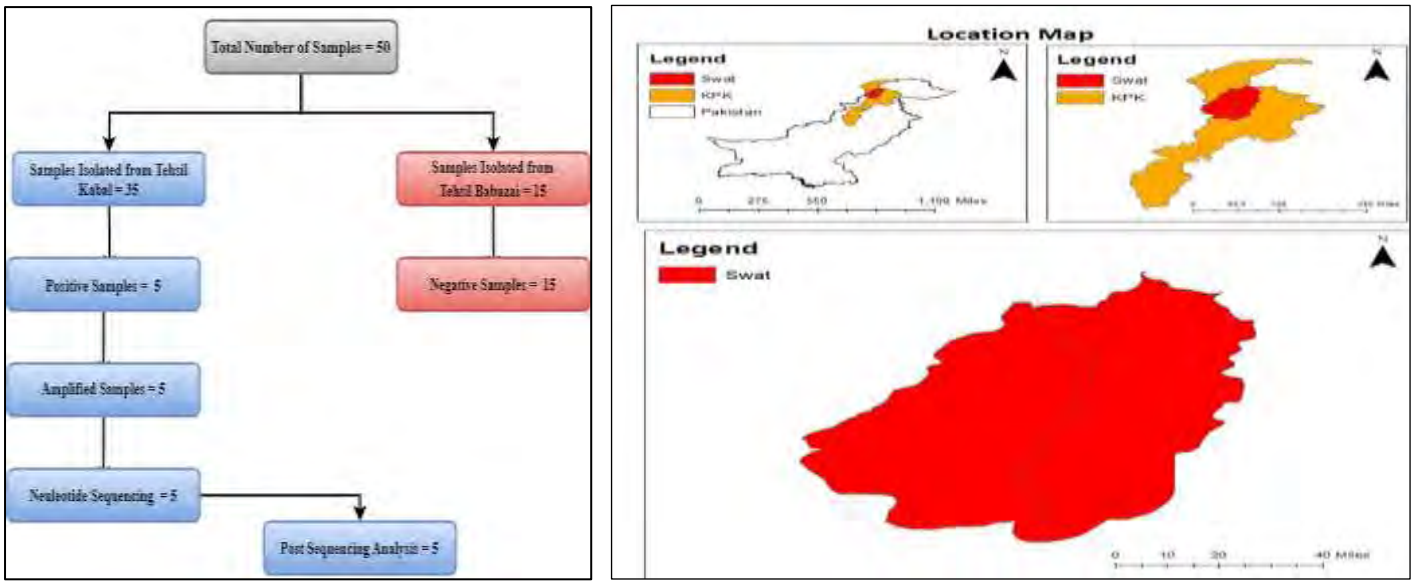


Figure 8 : Gel electrophoresis and visualization of amplified products (A) Lane 1 represent100bp ladder while Lane 2 and Lane 3 represent amplified PCR products of ~229bp (B) Lane 1 represent 100bp ladder while Lane 3, Lane 4 and Lane 5 represent amplified PCR product(C) Samples collection and processing flowchart (on lift) in which the samples from District Swat Khyber Pakhtunkhwa were collected from fruit farms.

4.2 Sequencing of the confirmed influenza virus isolates

Following amplification, each specimen was purified from the excised gel through a gel purification kit and then prepared for sequencing. The sequence was refined using the BioEdit or MEGA-11 program. In the current experimental investigation, 5 M gene sequences from bat associated Influenza virus were analyzed and evaluated using bioinformatics techniques. The chromatogram of nucleotide sequences is shown in Figure 9.

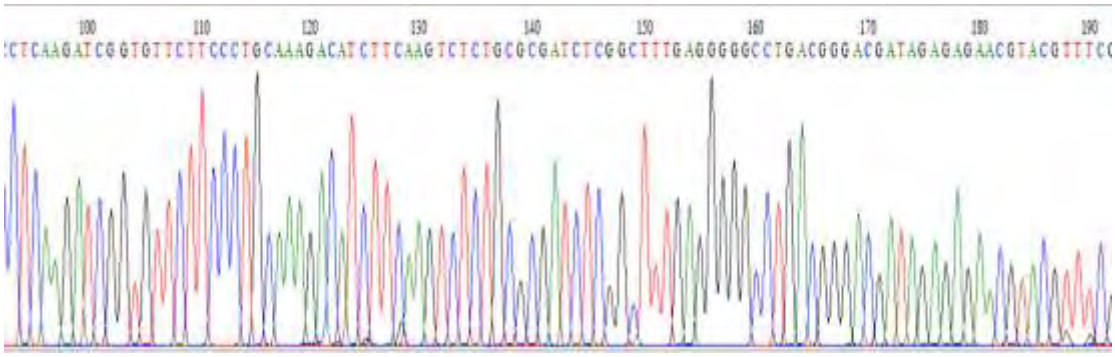


Figure 9 : Graphical representation of resulting nucleotide sequences of viral M gene in the form of chromatogram. Nucleotides A, T, G, and C are shown in red, green, black, and blue colors, respectively.

4.2 Evolutionary assessment of the bat influenza virus M gene sequences

To analyze the evolutionary relationship of the virus, we used the Neighbor-Joining approach to undertake a phylogenetic study of the acquired sequences with closely associated, previously published sequences.

Furthermore, Understanding the genetic variability, sequencing of the mentioned gene of Influenza virus samples was performed. A total of 50 reference sequences for 10 each isolate (QAU-HA2, QAU-HA-3, QAU-HA4, QAU-HA5 and QAU-HA9) had been acquired from NCBI database (GenBank). The evolutionary relationships among the study sequences and reference sequences were assessed using the neighbor-joining method in MEGA-11software package. A proportion of trees is shown Figure 10 next to the branches, where the similar taxa have been grouped together. The study demonstrates that influenza M gene isolates sequences of the current study QAU-HA2 make a cluster with previously reported sequence from Russia (accession number. KF462347) and QAU-HA5, QAU-HA4, QAU-HA3, QAU-HA9 make a cluster with previously reported sequence from Netherland (accession number. MF145856) which suggests a potential relationship among the viruses circulating in these countries. The taxa of current study viral isolates (n = 5) are labelled as red, while reference isolates (n = 50) of Influenza M gene, mostly from Russia, Netherland, Korea, Sweden and Nigeria and other regions are presented in black color as shown in the Figure 10.

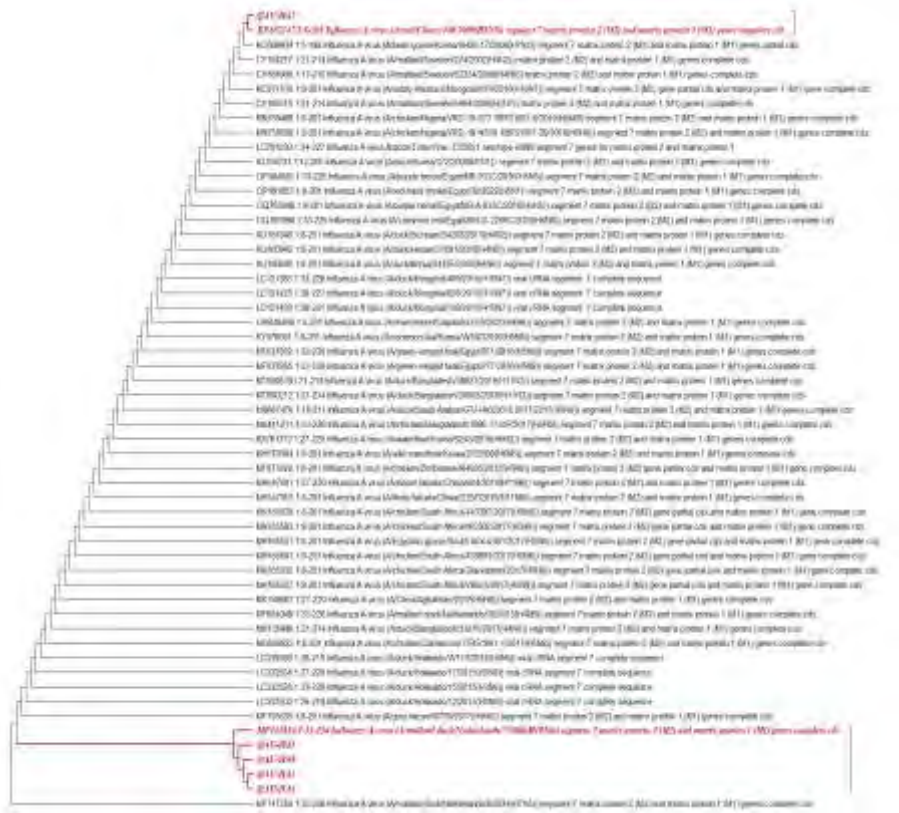


Figure 10 : Phylogenetic tree of the M gene nucleotide sequences of studied isolates (n=5) with the other reference sequences (n=50) from different countries of the word. The current study viral isolates are named (QAU-HA2, QAU-HA5, QAU-HA9, QAU-HA3 and QAU-HA4) labelled as red, while reference sequences are represented in black color.

4.3 Identification and alignment of amino acid sequences of Influenza M gene

The nucleotide sequences of all isolates were translated to amino acid sequences using online server ExPASy, followed by alignment of resultant amino acid sequences with reference sequences through CLC workbench (Version 8) as shown in the Figure 11. The M gene reference protein sequence (Accession No. AAA43244) was retrieved from NCBI GenBank. Upon comparison with reference sequence, significant amino acid substitutions were found in all isolates. There are 3 amino acid substitutions found in M protein of QAU-HA2 isolates that are F63V, S69R and E70Q. Similarly, 7 amino acid substitutions such as L58Y, V62L, F63G, T64S, L65R, T66S, and S69G were found in QAU-HA9 isolate. Moreover, in isolate QAU-HA3, 7 amino acid substitutions were observed that are E27Q, P52R, L53S, P54T, K55Y, I57D, and S69A. furthermore, there are 15 amino acid substitutions in QAU-HA5 Isolate, E27Q,

P52T, L53S, T54D, K55Y, I57D, L55F, G56R, F61W, F63H, T64V, T66P, V67P, S69P and E70K. In Isolate QAU-HA4 having 10 amino acid substitution, E27H, A39G, W43R, P52R, L58Y, L65V, T66H, V67A, P68H, and E70Q. Detailed information of amino acid substitutions of all isolates with reference sequence are given in Figure 11 and Table 5, 6 and 7.

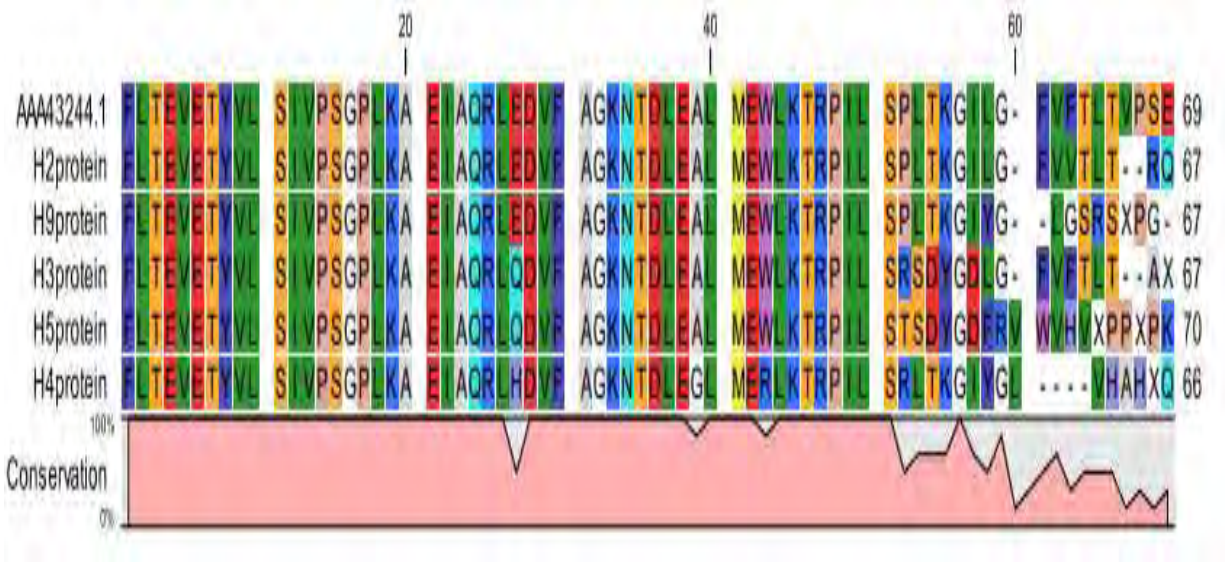


Figure 11 : Representation of the alignment of amino acid sequence of our isolates with reference sequence (AAA43244).

Table 7 : Amino acid substitutions in the Influenza isolate (QAU-HA2) Protein compared with the reference sequence of influenza M protein (AAA43244).

S.No.	Amino acid position	Wild type	Charge	Polarity	Variant	Charge	Polarity
1	63	Phenylalanine	Neutral	Non-polar	Tyrosine	Neutral	Polar
2	69	Serine	Neutral	Polar	Arginine	+ve	Polar
3	70	Glutamic acid	-ve	Polar	Glutamine	Neutral	Polar

Table 8 : Amino acid substitutions in the Influenza isolate (QAU-HA9) Protein compared with the reference sequence of influenza M protein (AAA43244).

S.No.	Amino acid position	Wild type	Charge	Polarity	Variant	Charge	Polarity
1	58	Leucine	Neutral	Non-polar	Tyrosine	Neutral	Polar
2	62	Valine	Neutral	Non-polar	Leucine	Neutral	Non-polar
3	63	Phenylalanine	Neutral	Non-polar	Glycine	Neutral	Non-polar
4	64	Threonine	Neutral	Polar	Serine	Neutral	Polar
5	65	Leucine	Neutral	Non-polar	Arginine	+ve	Polar
6	66	Threonine	Neutral	Polar	Serine	Neutral	Polar
7	69	Serine	Neutral	Polar	Glycine	Neutral	Non-polar

Table 9 : Amino acid substitutions in the Influenza isolate (QAU-HA3) Protein compared with the reference sequence of influenza M protein (AAA43244).

S.No.	Amino acid position	Wild type	Charge	Polarity	Variant	Charge	Polarity
1	27	Glutamic acid	-ve	Polar	Glutamine	-ve	Polar
2	52	Proline	Neutral	Non-polar	Arginine	+ve	Polar
3	53	Leucine	Neutral	Non-polar	Serine	Neutral	Polar

4	54	Threonine	Neutral	Polar	Aspartic Acid	-ve	Polar
5	55	Lysine	+ve	Polar	Tyrosine	Neutral	Polar
6	57	Isoleucine	Neutral	Non-polar	Aspartic acid	-ve	Polar
7	69	Serine	Neutral	Polar	Alanine	Neutral	Non-polar

Table 10 : Amino acid substitutions in the Influenza isolate (QAU-HA5) Protein compared with the reference sequence of influenza M protein (AAA43244).

S.No.	**Amino acid position**	**Wild type**	**Charge**	**Polarity**	**Variant**	**Charge**	**Polarity**
1	27	Glutamic acid	-Ve	Polar	Glutamine	-Ve	Polar
2	52	Proline	Neutral	Non-polar	Arginine	+Ve	Polar
3	53	Leucine	Neutral	Non-polar	Serine	Neutral	Polar
4	54	Threonine	Neutral	Polar	Aspartic acid	-Ve	Polar
5	55	Lysine	+Ve	Polar	Tyrosine	Neutral	Polar
6	57	Isoleucine	Neutral	Non-polar	Aspartic acid	-Ve	Polar
7	58	Leucine	Neutral	Non-polar	Phenylalanine	Neutral	Non-polar
8	59	Glycine	Neutral	Non-polar	Arginine	+Ve	Polar
9	61	Phenylalanine	Neutral	Non-polar	Tryptopan	Neutral	Polar
10	63	Phenylalanine	Neutral	Non-polar	Histadine	+Ve	Polar
11	64	Threonine	Neutral	Polar	Valine	Neutral	Non-polar
12	66	Threonine	Neutral	Polar	Proline	Neutral	Non-polar
13	67	Valine	Neutral	Nonpolar	Proline	Neutral	Non-polar
14	69	Serine	Neutral	Polar	Proline	Neutral	Nonpolar
15	70	Glutamic acid	-Ve	Polar	Lysine	+Ve	Polar

Table 11 : Amino acid substitutions in the Influenza isolate (QAU-HA4) Protein compared with the reference sequence of influenza M protein (AAA43244).

S.No.	Amino acid position	Wild type	Charge	Polarity	Variant	Charge	Polarity
1	27	Glutamic acid	+ve	polar	histidine	+ve	Polar
2	39	Alanine	Neutral	Non-polar	glycine	neutral	Non-polar
3	43	Tryptophan	Neutral	Polar	Arginine	+ve	Polar
4	52	Proline	Neutral	Non-polar	arginine	+ve	Polar
5	58	Leucine	Neutral	Non-polar	tyrosine	neutral	Non-polar
6	65	Leucine	Neutral	Non-polar	valine	neutral	Non-polar
7	66	Threonine	Neutral	Polar	Histidine	+ve	Polar
8	67	Valine	Neutral	Non-polar	Alanine	neutral	Non-polar
9	68	Proline	Neutral	Non-polar	histidine	+ve	Polar
10	70	Glutamic acid	-ve	Polar	Glutamine	neutral	Polar

4.4 Primary and secondary structure predication

The primary and secondary structure of influenza M protein was predicted via PSIPRED online server. The server used bioinformatics approaches that attempt to determine the local primary and secondary structures of proteins based only on their amino acid sequence. The primary and secondary of Reference sequence comprises 18%Beta sheet, 8%%alpha helix and 62% coil as shown in figure 12. Influenza isolate QAU-HA2 has a beta sheet of 19%, alpha helix of 38% and coil of 43% as shown in figure 13. Similarly, isolate QAU-HA3 has beta sheet of 21%. Alpha helix of 37% and coil of 47% as shown in figure 14. Isolate QAU-HA4 has a beta sheet of 4%, alpha helix of 52% and coil of 44% as shown in figure 15. Isolate QAU-HA5 has beta sheet of 16%, alpha helix of 41% and coil of 43% as shown in figure 16. while isolate QAU-HA9 has a beta sheet of 8%, alpha helix of 27% and coil of 65% as shown in figure 17.

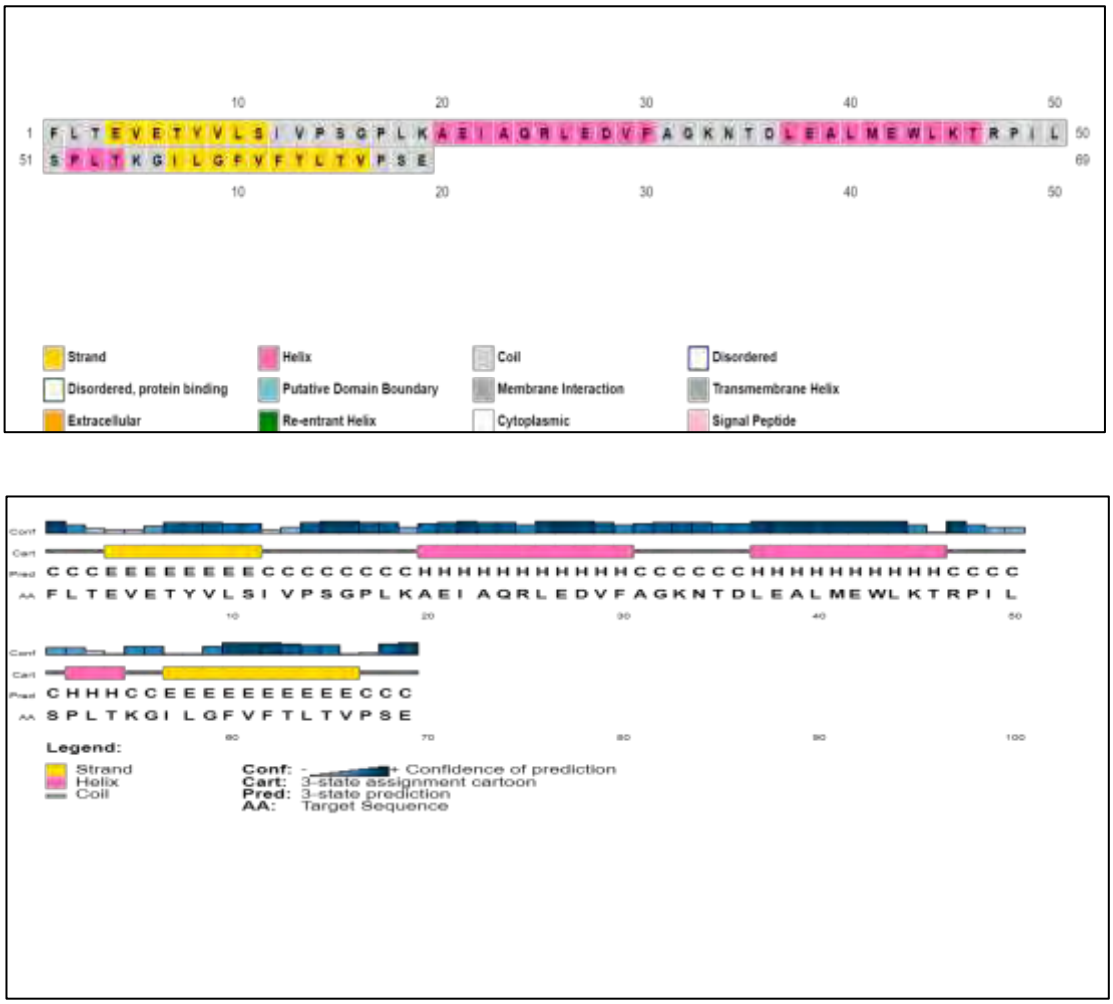


Figure 12 : Primary and secondary structure of the reference M protein sequences of Influenza A virus.Different motifs and region of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

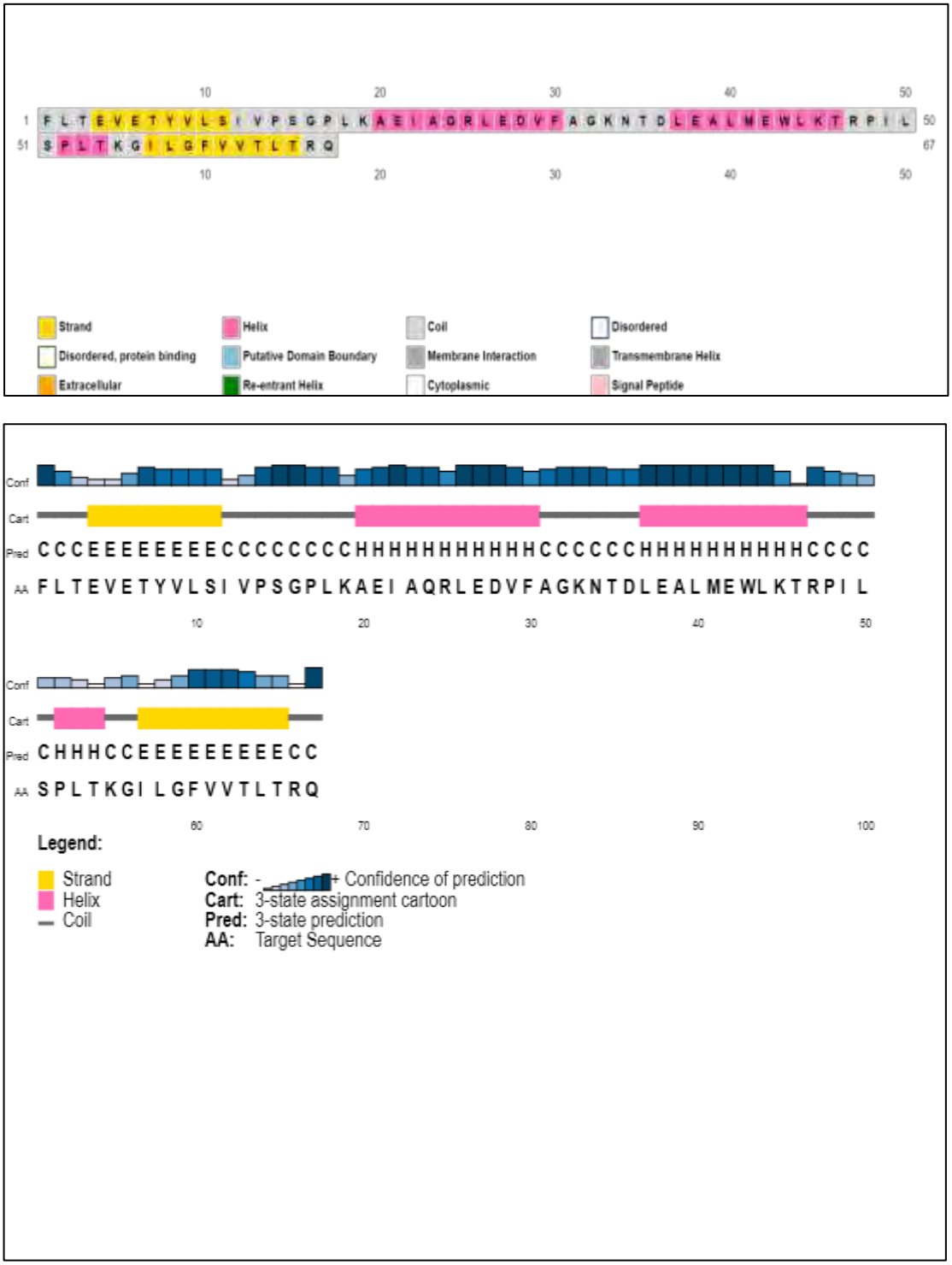


Figure 13 : Primary and secondary structure of M protein of QAU-HA2 isolate.Different regions and motifs of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

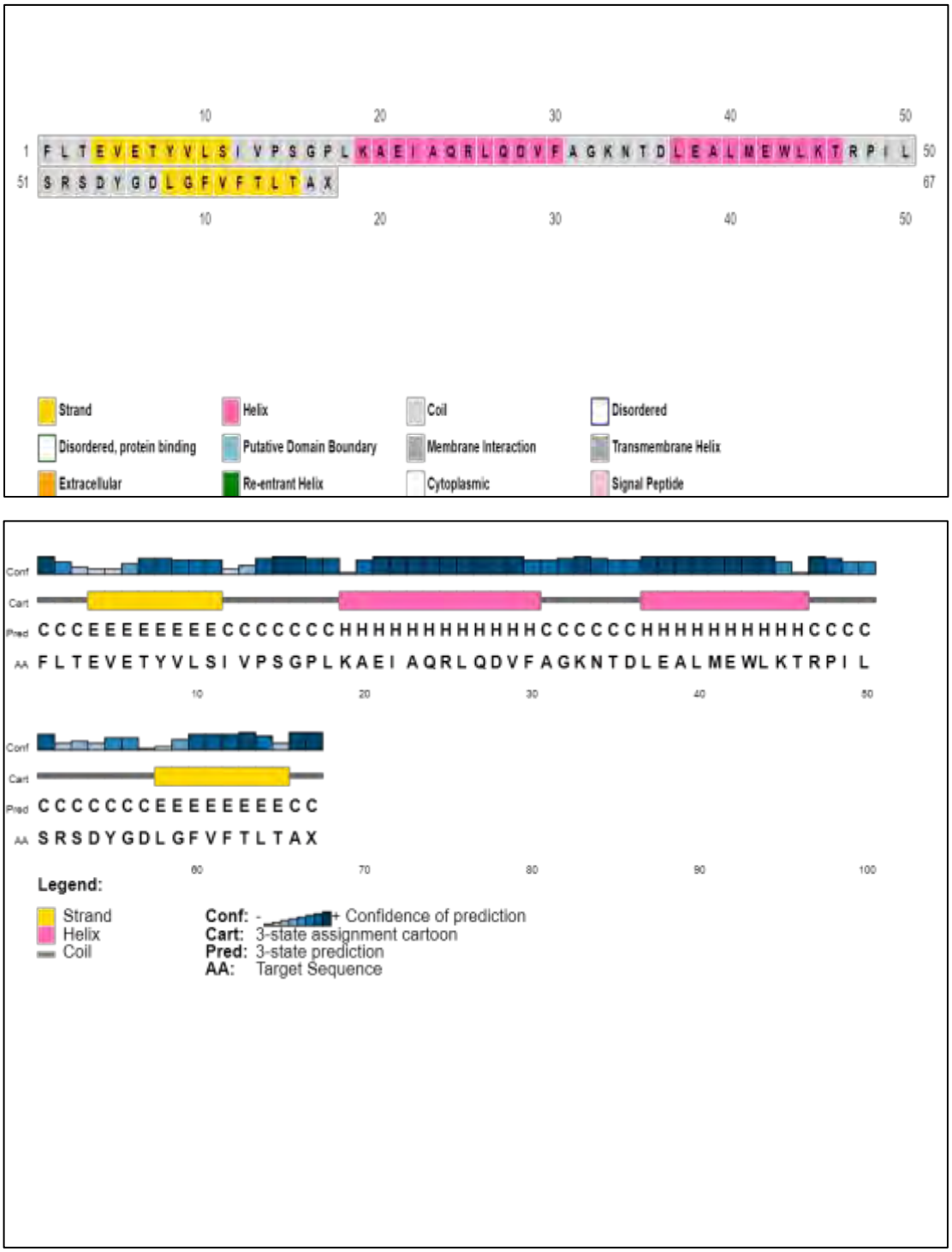


Figure 14 : Primary and secondary structure of M protein of QAU-HA3 isolate.Different regions and motifs of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

Figure 15 : Primary and secondary structure of M protein of QAU-HA4 isolate. Different regions and motifs of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

Figure 16 : Primary and secondary structure of M protein of QAU-HA5 isolate. Different regions and motifs of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

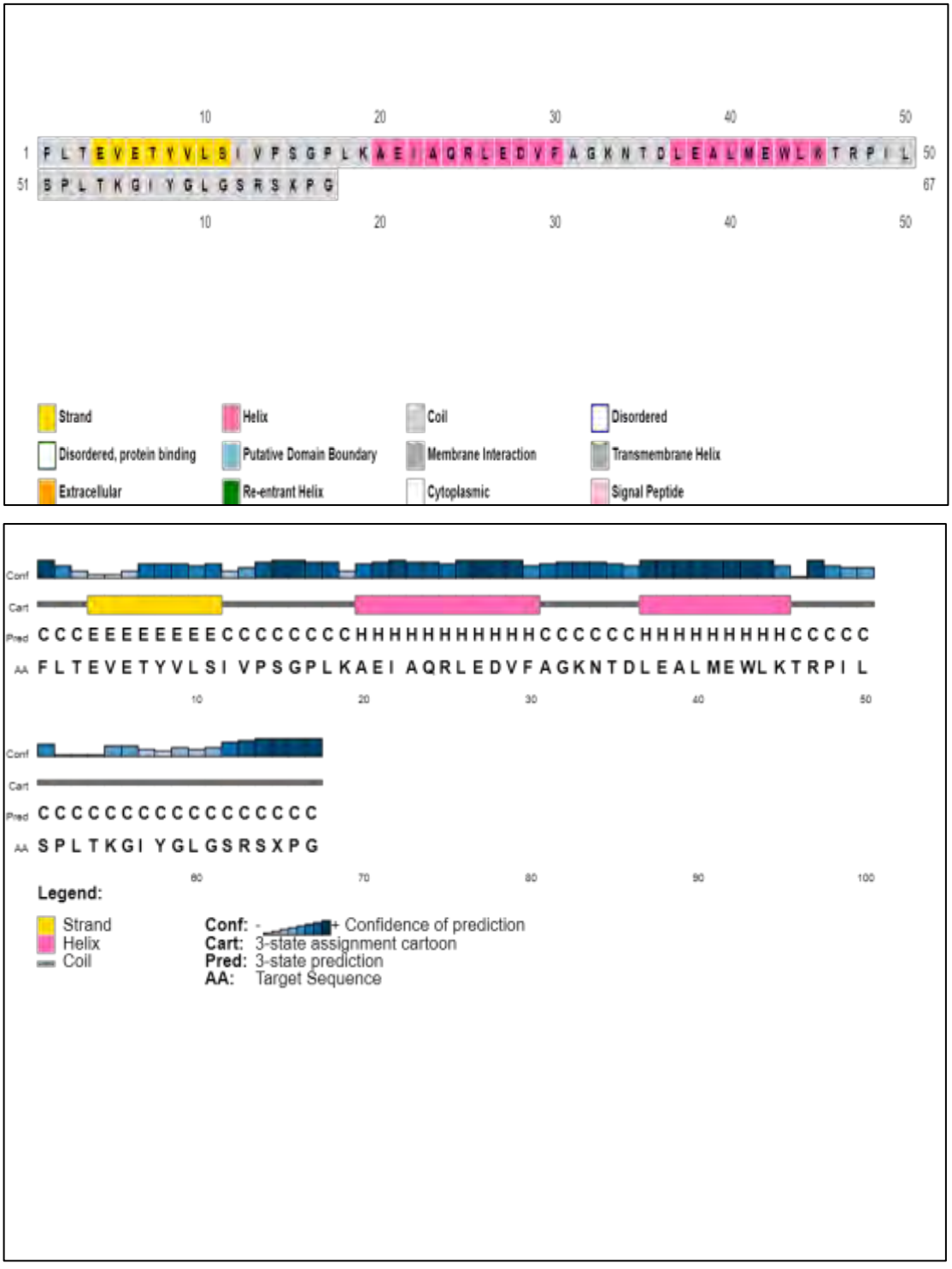


Figure 17 : Primary and secondary structure of M protein of QAU-HA9 isolate. Different regions and motifs of M protein such as, beta sheet, alpha helix and coil are represented in yellow, pink and light dark individually along with their target sequence length and percentages.

CHAPTER 05

DISCUSSION

5 Discussion

Approximately 1200 different species of bats exist, but only a tiny fraction of them have been tested for an outbreak of influenza virus. There is a strong likelihood that further virus samples along with potential IAV variants will be discovered (Mehle, 2014). Over 5% of the species of mammals are represented by bats, which are found almost everywhere on the globe. They act as natural hosts for certain viruses, such as the Influenza virus, Ebola virus, as well SARS-CoV (Calisher et al., 2006).

Over 60% of infectious diseases in humans are caused by pathogenic zoonotic agents (M. T. Rahman et al., 2020). At least four verified disease outbreaks, mainly influenza viruses, occurred in less than a century, despite the fact that a number of zoonotic viruses also significantly increased human deaths (Piret & Boivin, 2021). The most varied influenza A viruses, such as those with nine distinct copies of the NA gene and 16 different alleles of the HA gene, are thought to be present in wild populations of ducks (Tong et al., 2012).

IAVs can also infect many different species of host. Compared to flu viruses of canine, bats, bovine, and equines source, swine and avian influenza viruses have a higher risk for zoonotic spread (Abdelwhab & Mettenleiter, 2023). These viruses occasionally affect mammals, and in some instances, these infections result in persistent circulation in the new mammal hosts. Furthermore, Human can become infected with animal viral influenza despite the need for intermediary mammalian host. Beside pigs, several other animal species of animals, notably camels, cats, minks, dogs, cats, humans, seals, and numerous bird species, are viable mixing vessels carriers for the development of zoonotic animal viruses associated with influenza.

Moreover, previously reported research studies show that Nucleic acids recently found in bat specimens suggested that bats might be sources for a novel family of influenza viruses (bat-influenza) that are evolutionary extremely different from other influenza viruses (Tong et al., 2012). The influenza viruses that circulate in bats, H17N10 in the small yellow-shouldered bats while H18N11 in the flat-faced fruit-eating bats, are entirely novel variants that are historically separate from all other existing variants (Tong et al., 2012; Tong et al., 2013). Rectal swabs were used to

identify H17N10 initially, and then liver, kidney, intestines, and lung tissue while the H18N11 RNA was initially found in a rectal sample before being found only in intestinal tissue (Isaeva, Belkina, Rovnova, Kosiakov, & IaM, 1982; L'vov, Easterday, Hinshow, IuV, & Arkhipov, 1979).

Compared to any other influenza, the two variants are particularly highly connected to one another although they show a significant amount of variance (Tong et al., 2012). The possibility that these viruses, along with numerous other unusual influenza subtypes, could infect humans is a big worry. Although H17N10 and H18N11 contagious specimens or recombination forms have not yet been detected, tremendous progress has been achieved in comprehending the structure and function and ability of these viruses to replicate (Mehle, 2014).

Many animal groups, particularly populations of birds along with certain mammal species, have developed continuous transit of influenza type A infections. The set of mammals that influenza A viruses can live in may now include bats. Given that other bat-related viruses may use dogs, pigs, and horses as intermediary hosts. It is important to look into their capacity for allowing the spread of bat flu viruses (Tong et al., 2012).

Diagnostic techniques must be used to keep track of the viral strains that are currently circulating for influenza detection. Rapid influenza identification in a clinical or field context enables doctors and researchers to start treatment right away, put infection control measures in place, lower healthcare expenses, and lower hazards to patients, healthcare professionals, and the general public. Due to the influenza virus's continual evolution, diagnostic methods are frequently assessed and developed for higher specificity and validity such as virus isolation, molecular tests and serological tests (Swati Kumar & Henrickson, 2012; Wang & Taubenberger, 2010).

The creation of reliable molecular techniques that are non-invasive and enable accurate identification of influenza viruses linked to bats is crucial. In routine surveillance and epidemiological studies of the influenza virus, these can be extremely important. This study is the first from Swat, Pakistan to apply the non-invasive technique of RNA extraction from feces and amplifying M gene of influenza virus linked with bats (Viglino et al., 2016). we discovered, purified, and sequenced a

fresh IAV from guano bats fecal samples. 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 antigenic and glycosylation sites. Our research confirms the genetic variation of the influenza A(H3N8), (H4N6), (H7N3), and (H10N7) viruses and highlights the value of routine molecular surveillance for the efficient control of influenza pandemics and epidemics.

Conclusion

In the current study, the non-invasive procedure of guano bats fecal samples were analyzed for detection of M gene (partial) of influenza A. For this purpose, the target genome region was amplified and sequenced. Phylogenetic analysis suggests that the current study isolates QAU-HA2, QAU-HA5, QAU-HA9, QAU-HA3 and QAU-HA4) grouped together with sequences reported from Russia (KF462347), Netherland (MF145856),While shows up to 98.6% similarity with M gene in Nucleotide BlAST. Further studies are required for comprehensive surveillance throughout. our findings support the fact that Influenza A Virus are propagating within Pakistan's bats populations. Further research is required to examine the possibility for zoonotic viruses to spread between different species.

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