T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

MOLECULAR CHARACTERIZATION AND ISOLATION OF AVIAN INFLUENZA A VIRUSES FROM WILD AQUATIC BIRDS IN GEDIZ DELTA

YAVUZ MERCAN

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

IZMIR-2020

TEZ KODU: DEU.IBG.MSc-2017850033

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Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü
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(-) ssRNA: negative-sense single-stranded RNA

°C: Celsius degree

µg: microgram

µl: microliter

µm: Micrometer

µM: micromolar

A/Ala: Alanine

APC: Antigen presenting cell

BC: Before Christ

bp: Base pair

C/Cys: Cysteine

cc: Cubic centimeter

CDC: Centers for Disease Control and Prevention

CEIRS: Centers of Excellence for Influenza Research and Surveillance

cm: Centimeter

CI: Confidence interval

COI: Cytochrome oxidase I

CRBC: Chicken Red Blood Cell

D/Asp: Aspartate

ddH_2O: Double-distilled water

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleoside triphosphate

dsDNA: Double-stranded DNA

dsRNA: Double-stranded RNA

E#: Egg passage number

E/Glu: Glutamate

ECE: Embryonated Chicken Egg

EDTA: Ethylenediamine tetraacetic acid

EID_{50}: 50% egg infective dose

F/Phe: Phenylalanine

F: Forward

FAO: Food and Agriculture Organization

G/Gly: Glycine

GD/18: A/Aquatic bird/Gediz Delta/1/2018 (H6N2)

GIP: Global Influenza Program

GISN: Global Influenza Surveillance Network

GISRS: Global Influenza Surveillance and Response System

gr: Gram

H/His: Histidine

HA: Hemagglutinin

HA0: Uncleaved form of HA protein

HA1: First subunit of HA protein

HA2: Second subunit of HA protein

HPAI: Highly pathogenic avian influenza

I/Ile: Isoleucine

IAV: Influenza A virus

IBV: Influenza B virus

ICV: Influenza C virus

IDV: Influenza D virus

IFN: Interferon

K/Lys: Lysine

KU: Kilo unit

L/Leu: Leucine

LPAI: Low pathogenic avian influenza

M/Met: Methionine

M: Matrix

M1: Matrix-1 protein

M2: Matrix-2 protein

mg: Milligram

ml: Milliliter

mm: Millimeter

mRNA: Messenger RNA

MU: Million units

N/Asn: Asparagine

NA: Neuraminidase

NEP/NS2: Nuclear export protein/non-structural protein 2

ng: Nano gram

NIAID: National Institute of Allergy and Infectious Diseases

NIH: National Institutes of Health

nm: Nanometer

NP: Nucleoprotein

NS1: Non-structural protein 1

P/Pro: Proline

PA: Polymerase acidic protein

PB1: Polymerase basic protein 1

PB2: Polymerase basic protein 2

PBS: Phosphate Buffered Saline

PCR: Polymerase chain reaction

PBM: PDZ binding motif

pH: Power of hydrogen

Q/Gln: Glutamine

R/Arg: Arginine

R: Reverse

RdRp: RNA dependent RNA polymerase

RNA: Ribonucleic acid

rpm: Revolutions per minute

RT-PCR: Reverse-Transcriptase Polymerase Chain Reaction

S/Ser: Serine

SA: Sialic acid

T/Thr: Threonine

TAE: Tris-Acetic acid-EDTA

US: United States

V/Val: valine

vRNA: Viral RNA

vRNP: Viral ribonucleoprotein

W/Trp: Tryptophan

WHO: World Health Organization

Y/Tyr: Tyrosine

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I would like to dedicate this thesis to my grandparents, Haydar Mercan and Şadiye Güvenç who are not with us anymore, for being perfect role models for me. I will always try to live up to your examples and make you proud.

ABSTRACT

Molecular Characterization and Isolation of Avian Influenza A Viruses from Wild Aquatic Birds in Gediz Delta

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Due to its location, Turkey is visited by thousands of birds from different origins annually. Coexistence of these birds in wetlands contributes to Influenza A virus (IAV) evolution. Gediz Delta, provides a great opportunity for avian IAVs to evolve and adapt to mammals, as it hosts many different aquatic bird and mammalian species together. Thus, this thesis study aims to determine the prevalence of avian origin low pathogenic IAVs in Gediz Delta and to investigate the molecular markers in their genomes that may provide ability for interspecies transmission and to cause an outbreak. Additionally, phylogenetic analyses were performed to identify the origins of antigenic segments. To achieve this aim, 500 fecal samples of wild aquatic birds were collected in Izmir Bird Paradise/Gediz Delta. The samples were examined to detect IAV prevalence in the Delta via molecular methods. IAV-positive samples were inoculated into embryonated chicken eggs for virus isolation. Whole genome analyses were performed to monitor virus evolution. According to the results of this study, the prevalence of IAVs in Izmir Bird Paradise/Gediz Delta was 31.2% based on matrix gene amplification. In this thesis study, four viruses were isolated and the subtype of one virus was determined as H6N2. Whole genome analyses of H6N2 virus revealed 30 molecular markers which confers potential for zoonosis and adaptation to poultry. Phylogenetic analyses indicate that antigenic genes were originated from Asia. The results of this study indicate IAVs circulating in the Delta carry molecular markers which pose a threat for public and veterinary health.

Keywords: aquatic birds, low pathogenic IAVs, prevalence, molecular markers, zoonosis potential

ÖZET

Gediz Deltası'ndaki Yabanıl Su Kuşlarından İnfluenza A Virüslerinin İzolasyonu ve Moleküler Karakterizasyonu

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Türkiye, bulunduğu konum itibarı ile her yıl binlerce kuş tarafından ziyaret edilmektedir. Farklı kökenlerden gelen bu kuşların sulak alanlarda bir arada bulunması, influenza A virüslerinin (IAV) evrimine ve yayılmasına katkı sağlamaktadır. Bu sulak alanlardan biri olan Gediz Deltası, farklı kuş türlerinin yanı sıra birçok memeliye de ev sahipliği yapmakta, böylece IAV'lerin memelilere adapte olması için gerekli ortamı sağlamaktadır. Dolayısıyla bu çalışmanın amaçları; deltadaki yabanıl kuşlar tarafından taşınan düşük patojenli IAV'lerin prevelansının saptanması, bu virüslerin genomundaki zoonoz ve salgın riski taşıyan belirteç mutasyonların tespit edilmesi, ayrıca virüslerin antijenik genlerinin coğrafi kökenlerinin belirlenmesidir. Bu bağlamda İzmir Kuş Cenneti/Gediz Deltası'nda konaklayan yabanıl su kuşlarının dışkılarından 500 adet örnek toplanmış, moleküler metotlar kullanılarak deltadaki IAV prevalansı saptanmıştır. Sonrasında virüs izolasyonu için IAV pozitif örnekler embriyolu tavuk yumurtalarına inoküle edilmiş ve bir virüsün tüm genom analizi gerçekleştirilmiştir. Çalışmanın sonucunda IAV'lerin İzmir Kuş Cenneti/Gediz Deltası'ndaki matriks gen amplifikasyonuna dayalı prevalansı %31.2 olarak tespit edilmiş ve dört adet virüs izole edilmiştir. Alt tipi H6N2 olarak belirlenen bir virüsün tüm genom analizi sonucunda, virus genomunda zoonoz ve kümes hayvanlarına adaptasyon riski taşıyan 30 farklı belirteç mutasyon tespit edilmiştir. Ayrıca yapılan filogenetik analizler virüsün antijenik genlerinin Asya kökenli olduğunu göstermiştir. Bu çalışmanın sonuçları, deltada sirküle eden IAV'lerin insan ve hayvan sağlığını tehdit eden mutasyonlar taşıdığını ortaya koymuştur.

Anahtar kelimeler: sucul kuşlar, düşük patojenli IAV, prevalans, belirteç mutasyon, zoonoz potansiyeli

1. INTRODUCTION

Influenza A viruses (IAVs) have been one of the major public concerns for the past two centuries, since they caused three pandemics in the 20th century and one in the 21st century so far. Although pandemics have killed more than 50 million people, sporadic outbreaks and seasonal epidemics cause morbidity and mortality in both human and animal populations every year.

Natural reservoirs of IAVs are wild migratory aquatic birds (Anseriformes and Charadriiformes), however, these viruses can transmit to different species causing mild to severe infections (Webster *et al.*, 1992; Kocer *et al.*, 2014). Rapidly changing nature of the virus genome facilitates such transmission events. The genome of the virus consists of eight single stranded negative sense RNA segments. Lack of proofreading mechanism of RNA dependent RNA polymerase (RdRp) makes IAV genome prone to point mutations that may lead to alteration in protein sequences. Additionally, segmented structure of the genome provides IAVs to exchange gene segments when more than one subtype/strain of the virus infect the same cell. The genome of the virus encodes for 10 to 12 proteins along with several other auxiliary proteins which are expressed only in some viruses (Webster *et al.*,1992, Chen *et al.*, 2001, Jagger *et al.*, 2012). Two of the IAV proteins which are present on the surface of the viral envelope, hemagglutinin (HA) and neuraminidase (NA), are used to identify the subtype of the virus as HxNy. In their natural hosts 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified so far. Combinations of these constitute 144 possible subtypes, some of which have not emerged yet.

Avian origin IAVs caused or contributed to the emergence of four pandemics. Spanish Flu (1918) pandemic virus was thought to be an avian H1N1 virus that was transmitted to humans (Taubenberger and Morens, 2006). Asian Flu (1957) and Hong Kong Flu (1968) were H2N2 and H3N2 viruses, respectively and both obtained a part of their genome from avian origin IAVs (Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989). Lastly, the 2009 pandemic virus was an H1N1 virus (pH1N1). In spite of its swine origin, a part of the genome of pH1N1 was related to avian IAVs (Smith *et al.*, 2009). Apart from pandemic viruses, avian origin IAVs of different subtypes are occasionally detected in humans. Although human to human transmission of these cases are limited, each IAV carries the potential to cause another pandemic. Thus, in order to monitor and prevent future outbreaks and pandemics, regular surveillance studies are crucial.

Mediterranean/Black Sea and West Asian/East African flyways are two of the main flyways for migratory birds that also intersect with several other flyways. In these interception points, birds from various flyways may transmit different IAVs to each other. Several branches of both flyways cross over Turkey which result in high prevalence of aquatic bird species in the wetlands which are wintering, breeding, and stopping over sites for aquatic birds. Gediz Delta is one of the wetlands visited by thousands of migratory birds. The Delta is inhabited by many different mammalian species, as well. This species richness in the Delta increases the risk of adaptation of avian origin IAVs to mammals, including humans. Thus, surveillance studies on IAVs circulating among aquatic birds in the Gediz Delta is important to understand virus evolution and to determine the risk of zoonosis.

The aim of this thesis study is to determine the prevalence of IAVs circulating in wild aquatic birds residing in Izmir Bird Paradise that is a part of Gediz Delta and to identify the molecular markers harbored in the genomes of the viruses. In order to achieve this aim, the steps listed below are performed:

1. Collection of fresh fecal samples from wild aquatic birds in Izmir Bird Paradise
2. Detection of avian IAV prevalence in Izmir Bird Paradise through molecular methods
3. Isolation of avian IAVs to perform a comprehensive genomic characterization
4. Determination of species origin of HA and NA segments through phylogenetic analyses
5. Identification of molecular markers in the virus genome, which may provide selective advantage to the virus

2. REVIEW OF THE LITERATURE

2.1. Influenza Viruses

Influenza viruses are members of the Orthomyxoviridae family and there are four different types (Influenza A, Influenza B, Influenza C, Influenza D viruses) identified so far.

Influenza A viruses (IAV) were first isolated in 1933 (Smith *et al.*, 1933) from a patient; however, it was later determined that their natural reservoirs are wild aquatic birds (Webster *et al.*, 1992; Olsen *et al.*, 2006). This means that the virus is able to cross species barriers and infect different species including humans. IAVs caused three pandemics in the 20th century and another pandemic in the 21st century, resulting in deaths of more than 50 million people. The striking feature of all pandemic IAVs is that they are all genetically related to avian origin IAVs. Amongst these pandemic IAVs, two subtypes (H1N1 and H3N2) still circulate in human populations and cause seasonal flu infections which affect 3-5 million people and are responsible for 290000 to 650000 deaths annually all around the world (World Health Organization-WHO, 2020). Due to their high mutation rates and zoonotic potential, as well as their potential to cause pandemics, IAVs are under close inspection with regular surveillance studies. **This group of viruses will be discussed more extensively in the following chapters.**

Influenza B viruses (IBVs) were first isolated and identified in 1940 when the isolated virus did not have the same antigenic properties with IAVs. IBVs are also a known cause of seasonal flu along with IAVs. Two antigenically distinct lineages of IBVs identified in 1987 and 1988 that were named as Victoria and Yamagata, respectively (Rota *et al.*, 1990). Apart from human infections, IBVs have been reported to be isolated from marine mammals which refers to a zoonosis potential (Osterhaus *et al.*, 2000, Bodewes *et al.*, 2013).

Influenza C viruses (ICVs) were first identified in 1947 (Taylor, 1949) and are not considered as a health threat to humans, since they cause only mild infections. The antibodies to this group of viruses are present in newborns, which are inherited from their mothers (Homma *et al.*, 1982). ICV also detected in swine and cattle (Yuanji *et al.*, 1983; Zhang *et al.*, 2018). Although it was suggested that ICV might be able to transmit between different species (Kimura *et al.*, 1997), this hypothesis has not been proven experimentally yet.

Influenza D viruses (IDVs) were first identified in 2011 in a pig population as a distant relative to ICV strain. The poor genetic similarity of the virus genome to ICV lead to the conclusion that this was a distinct influenza type (Hause *et al.*, 2013). In addition to swine, IDV was detected in several other species like sheep, goat and horses (Quast *et al.*, 2015; Zhai *et al.*, 2017; Nedland *et al.*, 2018). However, natural reservoirs of the virus are thought to be cattles (Hause *et al.*, 2014). Further studies on IDVs showed that the virus is able to infect guinea pigs and transmit between individuals through direct contact (Sreenivasan *et al.*, 2015). IDV antibodies were also detected in humans who are in close contact with infected cattle (White *et al.*, 2016). Thus, IDV also poses a zoonotic potential.

2.2. Influenza A virus

H1N1 and H3N2 IAVs are the main cause of seasonal flu infections affecting 3-5 million people around the globe annually. Apart from seasonal infections, IAVs caused four pandemics (1918 Spanish flu, 1957 Asian flu, 1968 Hong Kong flu and 2009 swine flu) so far. IAVs are also known to be able to infect a wide range of species including land and sea mammals, wild birds and poultry (Figure 1). Due to their interspecies transmission ability and pandemic potential, surveillance studies on IAVs are conducted regularly throughout the world. These surveillance studies would allow the identification of novel virus strains, as well as genetic markers for zoonosis and/or pandemic potential.

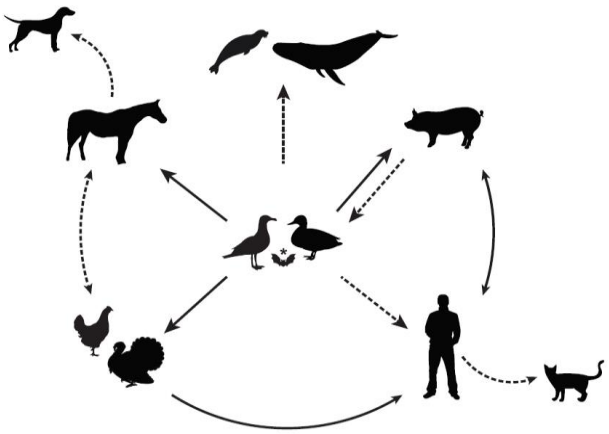


Figure 1. Host range of IAVs. Apart from their natural host, IAVs can infect poultry, swine, cats, dogs, horses, and humans. Rare routes for transmission are identified with dashed lines (Koçer *et al.*, 2014).

2.2.1. History of Influenza A virus and surveillance studies

There are many different opinions about the first occurrence of influenza in history. In 412 BC, Hipocrates described an illness, "fever of Perinthus", which resembles flu. However, the common opinion is that the first influenza outbreak emerged in 1580 according to the reports of physicians who described the disease (Potter, 2001). There are also several other reports describing influenza outbreaks in 1729, 1781, 1830 (Potter, 2001). Although these reports contributed to our knowledge on the disease, comprehensive research on influenza viruses gained momentum after the emergence of Spanish Flu (1918) and isolation of the first IAV from humans (Smith *et al.,* 1933). Studies on human isolates showed that antigenicity of the virus changes rapidly, which leads to decrease in vaccine efficiency and escape from immunity. Thus in 1947, Global Influenza Program (GIP) was started in collaboration with National Influenza Centers for data collection. Later in 1952, it was decided that surveillance efforts should also contribute to disease prevention and control; thus, Global Influenza Surveillance Network (GISN, later to be called GISRS), was founded.

First records of avian origin IAVs date back to the late 19th century, as a disease called fowl cholera was discovered (Alexander, 2003). However, it was not until the mid-20th century, the virus was found to be an IAV (Alexander, 2003). This discovery was followed by isolation of IAVs from avian species. First, an H5N1 virus was isolated from an infected chicken in Scotland (Pereira *et al.*, 1965), then an H5N3 virus was isolated from a tern in South Africa (Becker *et al*., 1966). Few years later, the possible role of migratory birds in dispersion of avian IAVs was pointed out (Easterday *et al.*, 1968). Later in 1973, another virus with H6N5 subtype was isolated from a shearwater species (Downie and Laver, 1973). This was followed by the isolation of 41 IAVs from ducks (Slemons *et al.*, 1974). After this date, surveillance studies on avian IAVs increased and led to the isolation and identification of 16 HA and 9 NA subtypes of IAVs from at least 105 different aquatic bird species (Olsen *et al*., 2006). Surveillance studies are regularly conducted all around the world to establish a better understanding of influenza prevalence and evolution. NIH/NIAID funds surveillance studies in the US and some other countries through the CEIRS program. There are also many other surveillance studies carried out in different countries. The Netherlands is one of the countries that place a great effort on influenza surveillance. In a study conducted in the Netherlands, around 153000 samples from wild birds and poultry were

collected for ten years between 2006 and 2016 (Bergervoet *et al.*, 2019). The results of the study indicate that the prevalence of IAVs was 0.88% in wild birds and 0.52% in poultry. The prevalence of virus subtypes was found to be different for each species and sampling time (season). Sequence analyses on antigenic segments (HA and NA) of IAVs detected in poultry showed that these segments were closely related with IAVs isolated from wild birds. This result indicates transmission of IAVs from wild birds to poultry (Bergervoet *et al.*, 2019). In another study, cloacal and fecal samples were collected for 8 years (2002-2010) from Öland, Sweden which is an important stopover site for migratory birds. The overall prevalence of IAVs was found to be 16.49%, ranging between 0% to 30%, depending on the season and migration periods of the wild birds (Latorre-Margalef *et al.*, 2014).

2.2.2. Surveillance studies in Turkey

Several branches of two main flyways, Mediterranean/Black Sea and West Asian/East African Flyways, cross over Turkey. As a result, thousands of migratory birds from different locations come to Turkey for wintering or stopping over for a period before they reach their wintering locations. Considering the role of migratory birds in dispersion of avian IAVs (Olsen *et al.*, 2006), wetlands where migratory birds reside along with many mammalian species, are hotspots for IAVs to reassort and/or to adapt to mammals.

The first report of avian IAV in Turkey was of a highly pathogenic H5N1 subtype during an outbreak which affected 54 cities and caused a severe economic loss (~32 million TL) between 2005-2008 (http://www.kusgribi.gov.tr/). The virus was first detected in samples collected from a turkey farm in Manyas, Balıkesir (Erturun, 2008). A group of researchers performed phylogenetic analyses on partial HA sequences that covers the cleavage site (220bp) of H5N1 viruses which were isolated during the outbreak (Ün *et al.*, 2008). This study illustrated that H5N1 viruses detected in Turkey were highly similar to the H5N1 viruses detected in neighboring countries. Additionally, viruses detected in Turkey were almost identical with H5N1 viruses detected in wild birds in China at the time. These results indicate that H5N1 viruses were possibly introduced to Turkey by migratory birds (Ün *et al.*, 2008). In another study, three proteins (PB2, HA and M) of H5N1 viruses which were isolated during the outbreak from humans and poultry were analyzed for molecular markers (Altıok *et al.*, 2008). They found mammalian adaptation markers in each of the analyzed proteins. PB2 protein of the viruses apart

from one chicken isolate harbored a well-known marker lysine (K) at position 627 of PB2 protein. HA proteins of one human isolate contained asparagine (N) at both positions 158 and 227 which provide increased affinity for mammalian type receptors. Each isolate carried valine (V) at position 28 of M2 protein which related with host specificity. In 2008, FAO in association with Turkish authorities evaluated the state of H5N1 outbreak in Turkey and gave recommendations on disease control and prevention (Newmann *et al.*, 2008). In another study, cloacal and tracheal samples from wild birds were collected in Kızılırmak Delta, Yumurtalık Lagoon and Nallıhan Bird Paradise (Çöven *et al.*, 2010). The overall prevalence was found to be 14.59%. Additionally, one virus with H12N2 subtype was isolated in this study. Another study was carried out in Kızılırmak Delta to detect IAV prevalence in wild birds. Analyses of 402 cloacal, tracheal and organ extract samples showed that the prevalence of IAVs was 0.49% (Albayrak and Ozan, 2010). In a study carried out between 2006-2009, fecal samples were collected at Van Lake basin, and overall prevalence was found to be 2.9%. From these positive samples, 12 viruses with subtypes H1N7, H7N9, H11N9, H8N4 and H5N1 were isolated (Boynukara *et al.*, 2011).

2.2.3. Influenza A virus classification

IAVs are named according to their surface glycoproteins, HA and NA. So far, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes were detected in avian species, which could allow the formation of 144 possible subtypes (H_xN_y). Apart from these, a few subtypes (H17 and H18; N10 and N11) were identified in bats using molecular methods, but these viruses could not be isolated so far (Tong *et al.*, 2012, Tong *et al.*, 2013).

All subtypes of IAVs naturally circulate in their natural hosts as low pathogenic avian influenza A viruses (LPAI), however the HA protein of some IAVs can acquire multibasic amino acid insertion at the cleavage site upon transmission to poultry. Resulting highly pathogenic avian influenza (HPAI) viruses can transmit to humans and may cause severe systemic infections with high mortality rate. Fortunately, human to human transmission of HPAIs are limited and very rare (Olsen *et al.*, 2005; Ungchusak *et al.*, 2005; Sun *et al.*, 2016). However, whether HPAIs would acquire efficient transmission ability among humans is still a major concern. Due to these

reasons, molecular markers related to transmission and virulence of HPAIs are studied extensively.

2.2.4. Genome of Influenza A Viruses

Genome of IAV is composed of eight single stranded negative sense RNA segments. Total length of the genome is around 13.5kb and encodes 10 to 12 proteins (Webster *et al.*,1992, Chen *et al.*, 2001, Jagger *et al.*, 2012) (Figure 2). There are also several other auxiliary proteins which are not expressed by all IAVs. In this section each gene and their protein products will be discussed.

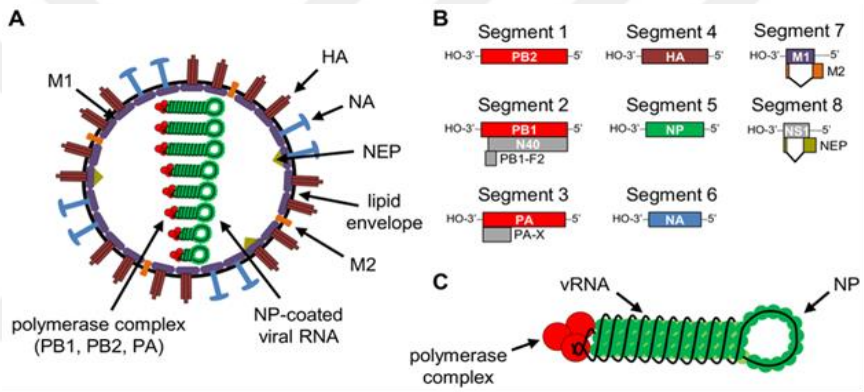


Figure 2. IAV genome and proteins. Cartoon representation of IAV virion (A), its genome with individual segments (B) and vRNP structure at which viral RNA is attached to NP proteins and polymerase complex (C) are shown (Heldt *et al.*, 2015).

2.2.4.1. Segment 1 (PB2 – Basic Polymerase 2)

PB2 protein is one of the three components of the viral polymerase complex that functions in cap snatching. This process is required for transcription, nuclear export and translation of viral mRNAs. Cap snatching involves binding of PB2 protein to 7-methylguanosine-containing cap (5'cap) of host mRNAs, which is cleaved by PA protein. After cleavage, a 10-20 nucleotide long host mRNA remains attached to the cap. This structure is used to prime transcription of viral mRNAs. Resulting viral transcript contains a cap that interacts with host factors to mediate nuclear export and translation. Additionally, 5'cap prevents viral mRNA from degradation. Uncapped pre-mRNAs are recognized by cap binding protein and degraded via non-sense mediated decay (Evdokimova *et al.*, 2001). Apart from its transcriptional roles, PB2 also plays a

role in immune evasion. PB2 protein interacts with mitochondrial antiviral signaling protein (MAVS) and inhibits interferon (IFN)-β expression (Graef *et al.*, 2010).

2.2.4.2. Segment 2 (PB1 – Basic Polymerase 1; PB1-F2; PB1-N40)

PB1 protein is the core subunit of the polymerase complex and interacts with both PB2 and PA proteins to mediate replication and transcription of viral RNAs (Webster *et al.*, 1992, Kobayashi *et al*., 1996) (Figure 3). It was shown that PB1 protein bears the polymerase activity and it is the minimal requirement for viral RNA transcription (Kobayashi *et al.*, 1996, Honda *et al.*, 2002). PB1 protein utilizes cap structures derived from the host with the functions of PB2 and PA, to prime the transcription.

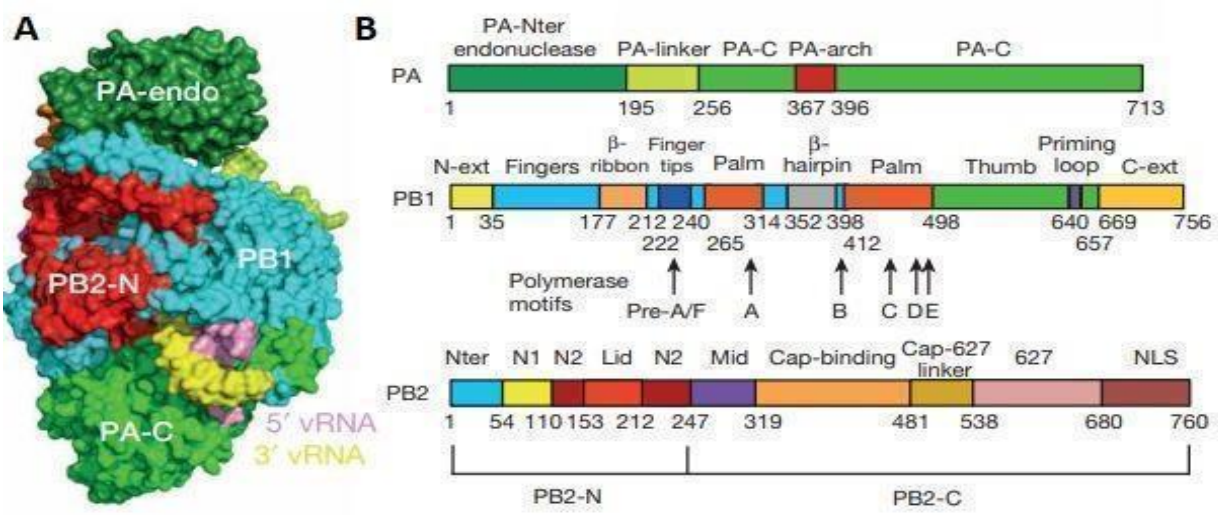


Figure 3. Side view space filling 3D structure of polymerase complex. PB1 (blue) protein interacts with both PB2 (red) and PA (green), there is no apparent direct interaction identified between PB2 and PA so far (A). Domains of polymerase complex subunits are also shown (B) (Pflug *et al.*, 2014).

i. Auxiliary proteins expressed from segment 2

PB1-F2 is another protein that is a product of segment 2 which is expressed through an alternative (+1) reading frame (Chen *et al.*, 2001). This protein was shown to induce apoptosis through interaction with mitochondrial proteins (Zamarin *et al*., 2005). The protein also contributes to the virulence, through promoting secondary bacterial infections (McAuley *et al.*, 2007). Apart from its role in virulence, PB1-F2 is also shown to increase polymerase activity

(Mazur *et al.*, 2008). Despite its roles and importance in pathogenesis, PB1-F2 protein is not expressed by all IAVs and is even dispensable for replication (Zamarin *et al.*, 2005). The most important example is the 2009 swine flu virus (pH1N1). The virus encodes a non-functional truncated version of the protein and introduction of the full length PB1-F2 had a limited effect on the virulence (Hai *et al.*, 2010).

The other protein product of segment 2, PB1-N40, was discovered in 2012 (Wise *et al.*, 2012). The protein is expressed due to alternative codon usage at the 5th starting codon of the segment 2 mRNA. Although no function has been appointed to the protein, computational analyses indicate that the protein may play a direct or regulatory role in viral replication, translation and several cellular events (Wang *et al.*, 2019).

2.2.4.3 Segment 3 (Acidic Polymerase protein-PA, PA-X)

PA is the acidic subunit of the viral polymerase protein which possesses endonuclease activity and plays a role in cap-snatching together with PB2 protein. PA cleaves host pre-mRNAs recognized by PB2 protein 11-13 nucleotides after 5' cap (Fodor *et al.*, 2002, Dias *et al.*, 2009). Resulting small capped host mRNA is then used to prime viral mRNA synthesis.

i. Auxiliary proteins expressed from segment 3

PA-X is another protein that is synthesized from segment 3 and is not expressed by all IAVs. The protein is translated due to +1 ribosomal frameshifting (Jagger *et al.*, 2012). Resulting protein takes part in shutting off host protein production by selectively degrading host mRNAs transcribed by RNA Polymerase II (Hayashi *et al.*, 2016). This process also limits the presentation of viral proteins by MHC-I to immune system cells and provides immune evasion (Jagger *et al.*, 2012).

Other two proteins, PA155 and PA182, are expressed from the 11th and 13th start codon of segment 3 mRNA, respectively (Muramoto *et al.*, 2013). These two proteins are N-truncated versions of the PA protein and thought to play a role in viral replication although their precise function is yet to be discovered. These two proteins are not expressed by all IAVs.

2.2.4.4. Segment 4 (Hemagglutinin-HA)

Segment 4 of influenza encodes major antigenic protein, HA, which is a surface glycoprotein taking part in both entry into the host cell and release of viral ribonucleoproteins (vRNPs). HA protein is expressed as a precursor polypeptide (HA0) which consists of two subunits, HA1 and HA2 (Figure 4). In its natural form, HA forms a homotrimer where each subunit consists of one HA1 and one HA2 protein (Wilson *et al.*, 1981). HA1 protein forms the globular head which is the major antigenic site of the virus. The head domain is responsible for virus binding to host cells through sialic acid (SA) receptors. The binding site consists of three conserved structures; 130-loop, 220-loop, and 190-helix (Ha *et al.*, 2001). HA2 contains the fusion peptide which mediates the release of vRNPs. This peptide is hindered in the natural form. Cleavage of HA0 enables fusion peptide to be revealed.

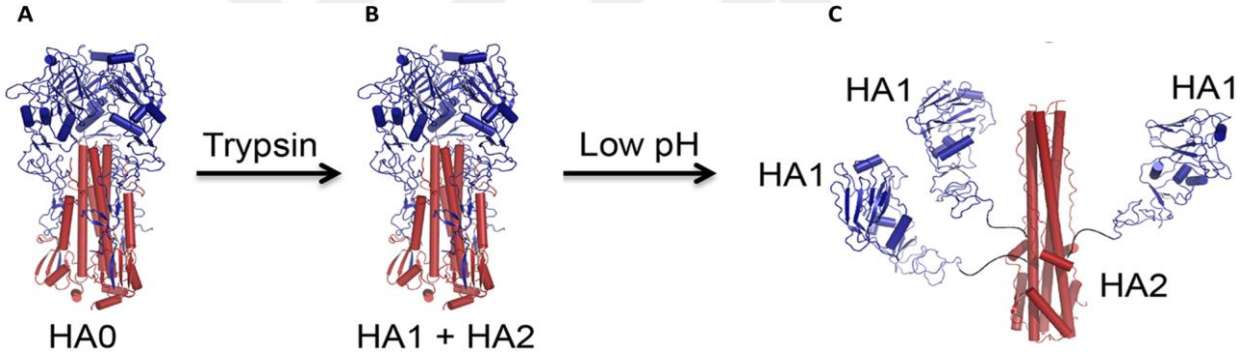


Figure 4. Change in HA structure during infection. HA0 molecule (A) is cleaved by host proteases at the cleavage site to form HA1 and HA2 (B). In the late endosome, conformation of HA molecule is altered due to a decrease in pH which is necessary for fusion and release of vRNPs (C) (Adapted from Chai *et al.*, 2016).

Entry into the host cell is initiated by attachment of HA protein to the SA receptors found on the host cell. After attachment to the host receptor, HA0 is cleaved by cellular proteases at the cleavage site (/GLF). In LPAI viruses, one basic amino acid (R or K) is located at amino acid position 329, right upstream of the cleavage motif (Wiley and Skehel, 1987). This motif at the cleavage site is targeted by trypsin-like proteases found in the respiratory and digestive tracts of the host. However, upon transmission to poultry, multibasic amino acid insertion into the cleavage site may occur (Figure 5). Such genetic changes at the cleavage site allows HA protein

to be cleaved by ubiquitous furin-like proteases. These proteases can be found in multiple tissues (brain, heart, liver etc.), thus the infection becomes systemic.

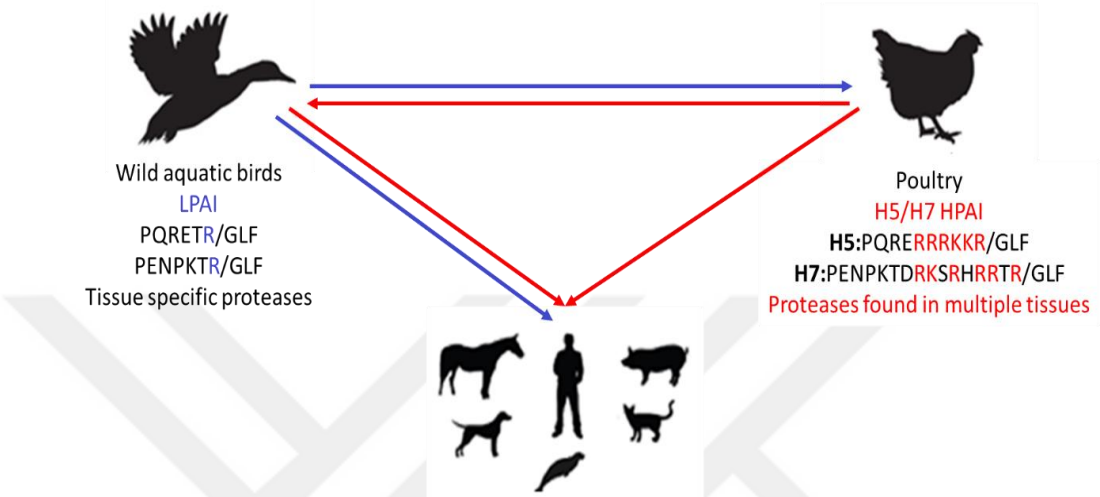


Figure 5. Conversion of LPAI viruses to HPAI viruses. LPAI viruses from wild aquatic birds can infect poultry and acquire multiple basic amino acids at the cleavage site of HA protein that allows the virus to become HPAI. The HA cleavage motif of LPAIs are recognized and processed by proteases found in the respiratory tract and digestive tract. On the other hand, the HA cleavage motif of HPAIs are targeted by proteases found in multiple tissues.

2.2.4.5. Segment 5 (Nucleoprotein-NP)

NP is a protein that is found in the RNP core. It is associated with viral RNA and protects it from intracellular nucleases. The major role of the protein is, however, to interact with viral polymerase complex and take part in transcription and replication of the viral genome (Honda *et al.*, 1988, Yamanaka *et al.*, 1990, Biswas *et al.*, 1998). NP protein also mediates nuclear transport of vRNPs. The protein interacts with cellular factors through two nuclear localization signals located at the N-terminal domain and between the positions 327-345 (Neumann *et al.*, 1997).

2.2.4.6. Segment 6 (Neuraminidase-NA)

NA protein is the second major antigen of IAVs found on the surface of the virus envelope. Main role of NA is to cleave SA residues from the host cells to prevent accumulation of newly formed viruses. This activity of NA is required for release of newly formed viruses from

the host cells (Palese *et al.,* 1974). In addition to its late phase role, NA also takes part in early stages of influenza infection. Human airway epithelium is covered with a protective mucus layer which is composed of glycosylated mucins. NA is shown to cleave SAs from mucins and improves the motility of the virus in the mucus to reach epithelial cells (Matrosovich *et al.,* 2004, Cohen *et al.,* 2013).

2.2.4.7. Segment 7 (Matrix proteins-M1 and M2)

Segment 7 of the influenza genome encodes for two proteins; M1 and M2. M1 protein forms the capsid structure which envelopes nucleoprotein complex (Figure 2A). M1 also interacts with other viral proteins and host factors for transportation of vRNPs to plasma membrane, and assembly of new virions. M2 is expressed as an alternative splice variant of segment 7. M2 is an integral membrane protein that serves as an ion channel. In the late endosome it decreases the pH of the virion interior. This change in the pH disrupts protein-protein interaction and leads to dissociation of viral capsid (Holsinger *et al.,* 1994; Alvarado-Facundo *et al.,* 2015).

i. Auxiliary protein expressed by segment 7

Segment 7 encodes for another protein, M42 which is not necessary for replication of IAVs. This protein is an alternative splice variant of matrix gene and was shown to have similar functions with M2 protein as it can compensate for the loss of M2 protein to some extent (Wise *et al.,* 2012). However, M42 protein differs from M2 protein by its cellular localization during infection. M2 protein localizes at the plasma membrane but M42 mostly localizes at cis-golgi apparatus (Wise *et al.,* 2012).

2.2.4.8. Segment 8 (NS1 and NS2)

Segment 8 encodes for two proteins, NS1 and NS2 (NEP), as a result of alternative splicing. NS1 protein has shown to be related with survival of the virus by antagonizing the interferon-mediated antiviral response of the host (García-Sastre *et al.,* 1998). The other protein expressed from the segment 8 is NS2 (NEP). This protein is mainly responsible for export of vRNPs from the nucleus (O'Neil *et al.,* 1998).

i. Auxiliary protein expressed by segment 8

Another protein, NS3, is also expressed by segment 8. Expression of this protein is a result of a mutation that alters adenine nucleotide to guanine at the position 374. This mutation leads to amino acid change at D125G as well as introducing a splice site for the transcript. Due to this mutation a new splice variant of segment 8, NS3, is expressed. Expression of this protein is related to the host switch of influenza from avian to mammalian species (Selman *et al.*, 2012).

2..2.5. Infection cycle of influenza A viruses

Infection cycle of IAVs composed of three main steps. 1) Virus entry and release of the viral genome, 2) Replication, transcription and translation, and 3) Assembly and release of newly formed virions (Figure 6).

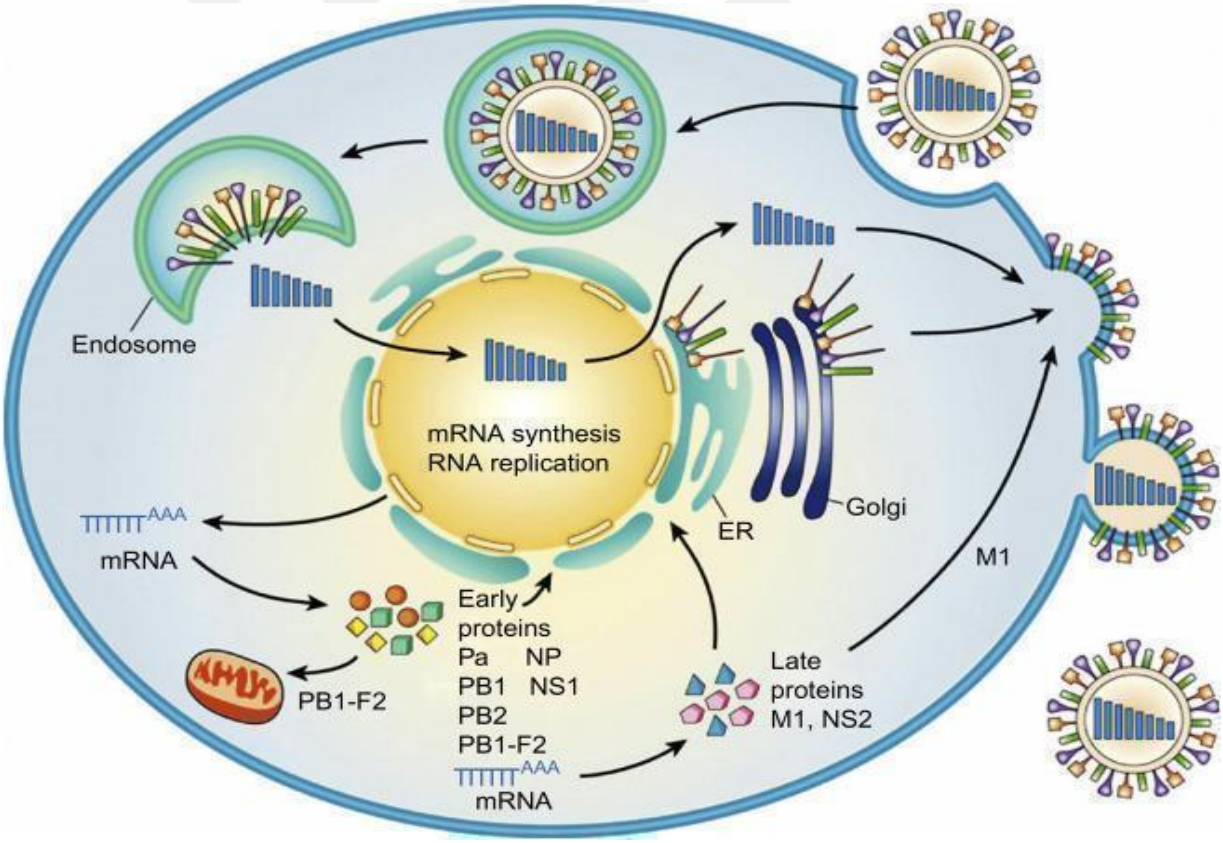


Figure 6. Summary of IAV infection cycle. IAVs attach to the surface of the host, internalized through receptor mediated endocytosis. vRNPs released to cytoplasm and transferred to the nucleus where both transcription and replication takes place. Viral mRNAs carried to the cytoplasm for translation. In early phases, proteins are transferred back to the nucleus to perform

their functions in replication, transcription or export of vRNPs and viral mRNAs. After vRNPs and viral proteins are localized at the periphery of the plasma membrane, budding and release of newly formed viruses takes place (Arias *et al.*, 2009).

2.2.5.1. Virus Entry

Infection cycle of IAV starts with the attachment of the virus to the host cell through the interaction between viral HA glycoprotein and host SA receptors. Upon interaction with the host SA receptor, HA molecule is cleaved by trypsin-like proteases and HA2 domain is revealed (Figure 4). Then, the virus is taken into the cell via clathrin dependent endocytosis. Although the main mechanism for IAV entry is clathrin dependent, clathrin independent endocytosis and macropinocytosis are also observed (Sieczkarski *et al.*, 2002; de Vries *et al.*, 2011). After internalization, fusion peptide in HA2 protein is revealed due to a decrease in pH in the late endosome. The fusion peptide fuses with the endosomal membrane while M2 channel protein decreases the pH in the viral envelope. This results in dissociation of capsid structure and release of vRNPs.

2.2.5.2. Replication, transcription, and translation

Upon dissociation of M1 protein, vRNPs are imported into the cytoplasm by host nuclear import proteins, importin-α and importin-β that recognize the nuclear localization signals found in NP (Wu *et al.*, 2007). Once the vRNPs localize into nucleus, a complementary RNA (cRNA) is transcribed for production of viral RNAs (vRNAs).

Transcription of viral mRNAs from vRNAs depends on the cap snatching which is mediated by PB2 and PA proteins (Fodor *et al.*, 2002, Dias *et al.*, 2009). The cap structures of host mRNAs are recognized by PB2 protein and cleaved by PA protein. Resulting 5'caps with short mRNA stretches are used to prime viral mRNA synthesis, thus the transcribed viral mRNAs possess a 5' cap. After maturation, viral mRNAs are transported to cytoplasm by host factors in association with NS1 protein to be translated by host translation machinery (York and Fodor, 2013; Pereira *et al.*, 2017). After viral mRNAs are transported to the cytoplasm, NS1 protein recruits cellular factors related to the translation process, such as eIF4F complex, for translation initiation (Yángüez and Nieto, 2011).

2.2.5.3. Assembly and release from the host cell

After translation, viral polymerase complex proteins, NP, NS1, NEP and M1 are transported back to the nucleus to carry out their functions (Bullido *et al.*, 2000; Deng *et al.*, 2006). On the other hand, M2, HA and NA are transported to the endoplasmic reticulum for post translational modifications. In the nucleus, NP and polymerase complex proteins associate with the newly formed vRNAs to form vRNPs. Then, vRNPs are exported to the cytoplasm by host factors in association with viral proteins M1 and NEP. In cytoplasm each vRNP is transported to the plasma membrane where they are packed together (Kawaguchi *et al.*, 2012). M2, HA, NA proteins are also transferred to the plasma membrane after they acquired necessary post translational modifications. The packaging occurs in 7+1 orientation where one vRNP is surrounded by seven others and requires vRNP-M1 interaction (Noda *et al.*, 2006). This interaction involves M1 binding to segment specific packaging signals on the vRNAs rather than NP protein (Nakatsu *et al.*, 2016, Bolte *et al.*, 2019). While vRNPs are being assembled, M1 starts to polymerize and budding is initiated at the periphery of lipid rafts where HA, NA and M2 proteins were localized (Rossman *et al.*, 2010). The budding is mediated by M2 protein, via altering membrane curvature in a cholesterol dependent manner. At the late stages of the budding M2 localizes at the neck of budding virion to complete the budding (Rossman *et al.*, 2010). After budding is complete, NA mediates the release of newly formed virions from the host cell surface (Palese *et al.*, 1974).

2.2.6. Host range and interspecies transmission of influenza A viruses

Avian IAVs are naturally carried by wild aquatic birds (Anseriformes and Charadriiformes) and in their natural hosts IAVs can remain quiescent, without any apparent symptoms. Through infected migratory birds, the virus can travel to great distances at which the virus can transmit to other migratory or local birds (Olsen *et al.*, 2006). Apart from their natural hosts, rapidly changing virus genome allows the virus to transmit and adapt to new hosts such as pigs, cats, dogs, horses, poultry, sea mammals and humans (Figure 1). It has been shown that IAVs which are isolated from avian species are able to infect mammals causing morbidity and mortality (Koçer *et al.*, 2012; Driskell *et al.*, 2012; Zanin *et.al.*, 2017a). The transmission of IAVs from avian hosts to humans can occur either directly (Zhang T *et al.*, 2014; Zhang W. *et al.*, 2014) or

through an intermediate host like swine upon undergoing adaptive mutations as in the case of swine flu pandemic (Shortridge *et al.*, 1977, Smith *et al.*, 2009).

Although all HA and NA subtypes were detected in aquatic birds, the other species are exclusively infected by specific subtypes of IAVs. For example, swine is shown to be infected extensively by H3N2, H1N1 and H1N2 subtypes. On the other hand, horses are infected mostly by H3N8 and occasionally by H7N7 viruses. Similarly, canine species are mostly infected by H3N2 viruses. Among the species within the host range of IAVs, poultry has one of the most diverse repositories in terms of IAV subtypes. Despite the fact that most of the IAV subtypes were detected in poultry, the most prominent subtypes are H5, H7 and H9 (Influenza Research Database IRD, www.fludb.com). The reason for the dominance of certain subtypes might be the bias towards those during IAV surveillance studies in poultry. Because H5, H7 and H9 are critical subtypes in terms of their effect on poultry industry. H5 and H7 subtypes are able to become highly pathogenic upon transmission to poultry. Once they become HPAI, they can transmit to humans and cause systemic infections with high mortality rates. H9 viruses have become endemic in poultry. H9 infections in poultry negatively affect egg yields thus, causing economic loss. Additionally, reassortment events between H9 and H5/H7 IAVs are detected frequently, which poses a public health risk.

Host preference of IAVs depends on the affinity of HA protein for different SA receptor types on the host cell membrane. HA of avian origin IAVs have a greater affinity for α- 2,3 linked SA receptor while HA protein of mammalian origin IAVs have a greater affinity for α-2,6 linked SA receptor (Figure 7). Mammalian upper respiratory tract epithelium cells equipped with α-2,6 linked SA receptor whereas lower respiratory epithelium cells, as well as avian digestive tract epithelium cells, possess α-2,3 linked SA receptor (Ito *et al.*, 1997; Nicholls *et al.*, 2007). Therefore, adaptation of IAVs to mammals requires a shift in antigenicity that alters preference of the protein for SA receptors. Activation pH of HA proteins of human adapted viruses are more acidic than of avian viruses (Di Lella *et al.*, 2016). HA stability at lower pH is required for virus motility in mucus that covers the human respiratory tract (Krenn *et al.*, 2011). Thus, a mutation that increases the stability of an avian origin virus for lower pH values can shift the host preference of avian IAVs to mammals (Mair e*t al.*, 2014; Di Lella *et al.*, 2016).

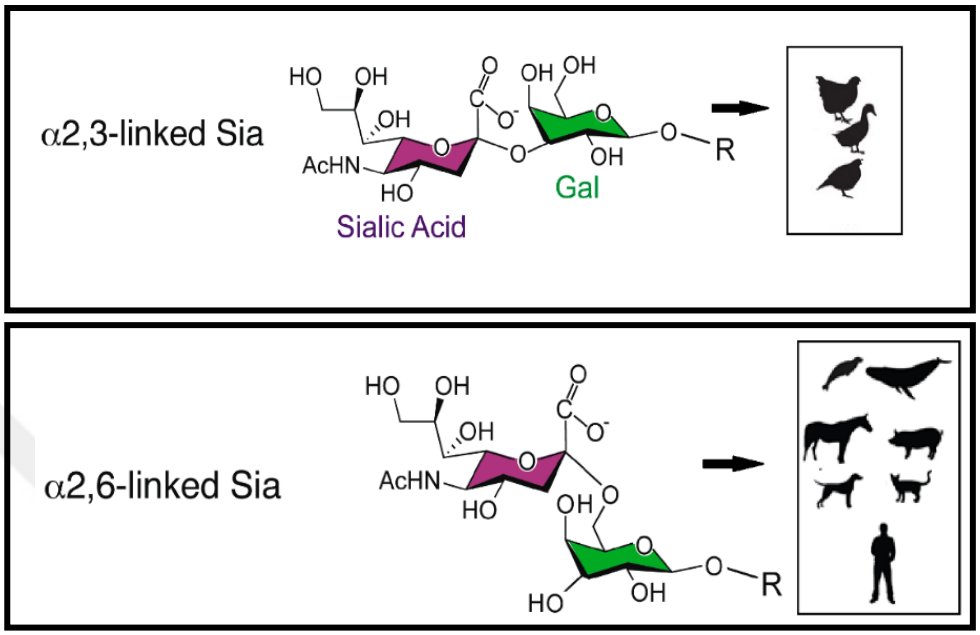


Figure 7. Receptors for IAVs. Main determinant in host preference of IAVs is affinity of HA for different SA receptors. HAs of avian and mammalian IAVs have a greater affinity for α-2,3 linked SAs and α-2,6 linked SA receptors, respectively (Adapted from Byrd-Leotis *et al.*, 2017).

Although the main determinant for the host preference is the HA protein, other viral proteins, such as polymerase complex proteins, also contribute to host switch. For example, genetic changes that promote polymerase complex of avian origin viruses to work efficiently in lower temperatures facilitate efficient replication in mammalian hosts (Hatta *et al.*, 2007). Apart from physical barriers, interaction of host proteins with polymerase complex is important for host determination. Mutations that alter specificity of polymerase complex for mammalian factors also play role in the host switch (Gabriel *et al.*, 2011).

2.2.7. Genetic Diversity of Influenza A Viruses

Broad host range and diversity of IAVs are results of rapidly changing nature of the viral genome. These genetic changes not only mediate interspecies transmission and adaptation to new hosts but also provide a selective advantage to the virus in terms of pathogenicity and replication efficiency.

2.2.7.1. Amino acid substitutions

RNA structure and lack of proofreading mechanism of RdRp result in frequent nucleotide changes which may result in amino acid substitutions. Some of these substitutions result in non-viable viruses, however some of them can provide a selective advantage to viruses (increased replication or transmission ability, adaptation to a new host, better immune evasion etc.).

Mutations in the polymerase complex proteins can increase replication ability of viruses and provide adaptation to new hosts. Genetic changes in HA protein can alter affinity of the protein for different SA, thus may alter host preference and/or provide immune evasion if it affects antigenicity of the protein. Mutations in NA protein can increase the virulence of viruses. Additionally, since NA is an antiviral drug target, mutations in the protein can result in drug resistance. Amino acid substitutions in NP, NS1, M1, M2, PB1-F2 and PA-X may increase replication, pathogenicity and contribute to transmission of IAVs. Some of the most studied amino acid substitutions are located on PB2 protein. For example, substitution of glutamate (glu, E) amino acid at 627^{th} position to lysine (lys, K) (E627K) (Subbarao *et al.*, 1993), or amino acid change, aspartate (asp, D) to asparagine (asn, N) at position 701 (D701N) (Li *et al.*, 2005) confers better replication ability in mammals. These substitutions are two of the most prominent mammalian adaptation markers. Substitutions at 226^{th} and 228^{th} position of HA protein are also examples of well-known mammalian adaptation markers. Q226L and G228S substitutions alter affinity of HA protein for SA receptors from avian to human (Rogers and Paulson, 1983).

In addition to mammalian adaptation, airborne transmission of avian IAVs is also a major concern. In that regard, NS1 protein was shown to be an important factor for not only disease pathology but also for the airborne transmissibility of IAVs. In one study E227G substitution in NS1 protein was shown to correlate with airborne transmission of the virus (Koçer *et al.*, 2015). Another study highlighted the importance of position 213 of NS1 protein. Serine (S) to proline (P) change in this position resulted in abrogation of airborne transmission (Zanin *et al.*, 2017b).

PB1-F2 protein which is a ribosomal frameshift product of segment 2 was shown to be determinant for pathogenicity as it functions to kill infected cells (Zamarin *et al.*, 2005). Amino acid substitution N66S in PB1-F2 protein was shown to increase pathogenicity of the virus, hence the disease severity.

2.2.7.2. Gene Exchange-Reassortment

Segmented nature of IAVs enables viruses to exchange gene segments between two different virus strains or subtypes when two or more IAVs infect the same cell. Gene exchange is one of the major concerns for public health as the phenomenon gave rise to at least three of the IAV pandemics (Kawaoka *et al.,* 1989; Lindstorm *et al.*, 2004, Smith *et al.*, 2009). Apart from pandemic viruses, reassortment events resulted in the emergence of many novel viruses. One example of such viruses is the H7N9 virus that emerged in China in 2013. The virus obtained its genes from three different avian viruses and transmitted to humans (Gao *et al.,* 2013). Another example for reassortant viruses is the H10N8 virus which caused human infections (Chen *et al.*, 2014). This virus obtained its six internal genes from avian IAVs circulating in poultry. Origin of HA and NA genes of the virus was IAVs detected in wild birds (Zhang T. *et al.*, 2014b).

2.2.7.3 Antigenic drift and antigenic shift

Amino acid changes on antigenic proteins (HA and NA) of IAVs are frequently detected due to selective pressure applied on these proteins by the immune system of the host organism. Accumulation of amino acid substitutions on these proteins may alter their antigenic characters. Such genetic changes are called antigenic drift (Figure 8). Antigenic drifts are the main reason for annual updates in vaccine components based on global IAV surveillance data, as they may significantly affect the vaccine efficiency. Antigenic drift can also delay immune response as the adaptive immune system of the host cannot recognize the altered antigen (Strengell *et al.*, 2011, Das *et al.*, 2013).

Larger and more drastic changes in antigenic sites are called antigenic shift (CDC, 2020b). Antigenic shift involves the reassortment of HA and NA segments (Figure 8), which may result in the emergence of novel IAV subtypes or strains. Resulting reassortant viruses can cause severe infections as the host immune system would be naïve for the new antigen.

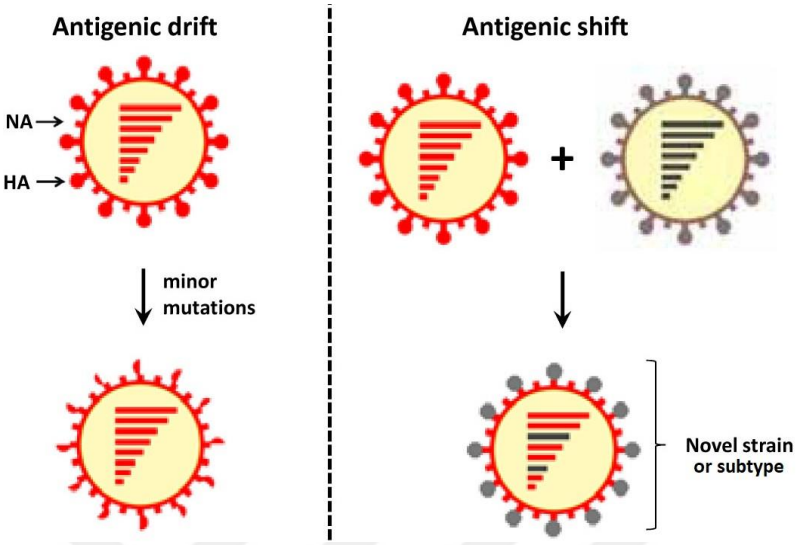


Figure 8. Antigenic drift versus antigenic shift. Antigenic drift is a result of accumulation of amino acid changes on HA and NA genes. Antigenic shift involves exchange of antigenic segments which results in emergence of novel IAV subtypes or strains (Mesquita *et al.*, 2011).

2.2.8. Emergence of pandemic viruses

IAVs are one of the major public health concerns as it caused 4 pandemics that killed more than 50 million people in the last two centuries (1918, 1957, 1968, 2009). Avian origin LPAIs contributed to the emergence of all pandemic viruses. In this section, the genetic basis for the emergence of these pandemic viruses will be discussed.

2.2.8.1. 1918, Spanish Flu (H1N1)

Spanish Flu started in 1918 and rapidly spread throughout the world. The pandemic affected 1/3 of the world's population and resulted in the death of approximately 50 million people (CDC, 2020c). This extensive spread was partly due to the impact of the First World War which led to poor hygiene practices, inadequate nourishment, and displacement of a huge number of people from war zones to other places. The viral RNA was successfully isolated in 1997 from frozen lung tissues of 5 different people who died in 1918 (Taubenberger *et al.*, 1997). Whole genome sequencing proved that the subtype was H1N1 and all genes of the virus originated from avian sources (Taubenberger and Morens, 2006). The HA protein of all isolates had E190V amino acid change which enables HA to shift binding preference from α-2,3 linked SA receptors

(avian) to α-2,6 linked SA receptors (mammalian). Additionally, although PB2 protein of the virus was clustered with avian viruses in phylogenetic analyses, it contained a lysine amino acid at the 627th position which is a well-known mammalian adaptation marker (Taubenberger *et al.*, 2005). Further studies also indicated that the high virulence of the virus was associated with NS1 protein (Basler *et al.*, 2001, Geiss *et al.*, 2002). Another determinant for the pathogenicity of 1918 virus was shown to be related with PB1-F2 protein. In a study, expression of PB1-F2 proteins of 1918 virus in a human IAV (PR8) increased the pathogenicity of the virus in mice (McAuley *et al.*, 2007). The virus established in the human population and continued to circulate as a seasonal flu virus.

2.2.8.2. 1957, Asian Flu (H2N2)

Approximately 40 years after the 1918 pandemic, a new pandemic emerged in Singapore in 1957 and quickly spread across the world killing more than 1 million people (CDC, 2020c). Subtype of the virus was H2N2 and it retained its five segments (PB2, PA, NS, M, NP) from the 1918 pandemic virus. The other three segments (HA, NA, PB1) were introduced to the virus from avian IAVs (Scholtissek *et al.*, 1978, Kawaoka *et al.*, 1989). PB2 protein of the virus contained a well-known human signature at the position 627 of PB2 protein (K627). H2N2 virus established and circulated in humans for 11 years till it was replaced by the 1968 pandemic virus. After that, the virus disappeared and has not been detected in the human population so far. However, H2N2 viruses have continued to circulate among wild birds and poultry (Jones *et al.*, 2014; Araujo *et al.*, 2017). It was also shown that avian H2N2 viruses were able to infect mammals and transmit via direct contact (Jones *et al.*, 2014). The transmission of H2N2 viruses to mammals in nature was also observed. An H2N2 virus was isolated from a muskrat and phylogenetic analyses showed that the virus was closely related to avian viruses (Gulyaeva *et al.*, 2017). Thus, H2N2 viruses still have the potential to transmit to humans and cause another pandemic, as immunity to H2N2 virus in humans is absent especially in people born after 1968 (Babu *et al.*, 2018).

2.2.8.3. 1968, Hong Kong Flu (H3N2)

A decade after Asian Flu, another pandemic arose in Hong Kong. The antigenic character of the HA protein of the new virus was completely different from previous human viruses, which indicated the emergence of a novel virus strain (Schulman and Kilbourne, 1969). The virus was

indeed a new strain, H3N2, that inherited its six genes (PB2, PA, NS, NA, M, NP) from the previous pandemic virus, H2N2, of which five segments originated from 1918 virus. Remaining two segments of H3N2 virus (HA, PB1) were shown to be acquired from avian origin viruses (Schulman and Kilbourne, 1969; Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989). PB1 protein of the 1968 virus differed from its avian counterparts by three amino acids R121, V212, and K327 which was shown to increase polymerase activity and viral replication in mammals (Wendel *et al.*, 2015). PB2 protein of the virus lacked mammalian marker at the position 627, however it harbored six other markers (V89, D309, K339, G477, V495, T676) which may compensate the effect of K627 (Li *et al.*, 2009). The variants of the virus still circulate in humans and cause seasonal infections.

2.2.8.4. 2009, Swine Flu (H1N1)

Latest IAV pandemic started in 2009 and it is estimated that the virus killed around 500.000 people (CDC, 2020c). The virus was shown to be of swine origin (Smith *et al.*, 2009). As it was proposed before, swine is a mixing vessel for avian and mammalian viruses as their upper respiratory tract contains both avian and mammalian types of SA receptors (Shortridge *et al.*, 1977, Scholtissek *et al.*, 1978, Webster *et al.*, 1992, Ito *et al.*, 1997). The genome of the virus was a combination of two different swine origin viruses (Figure 9). M and NA genes of the 2009 virus were obtained from a Eurasian avian-like swine H1N1 virus. Other six segments were acquired from a triple reassortant swine virus that inherited its genes from avian (PB2, PA), swine (HA, NP, NS) and human (PB1) origin IAVs (Smith *et al.*, 2009).

Genetic analyses indicated that the virus had amino acid changes that are related with its pathogenicity and transmissibility. The virus contained various amino acid markers for mammalian adaptation. Surprisingly, PB2 of the virus had avian signatures at 627^{th} and 701^{st} amino acid positions. However, it possessed a serine (S) at 590^{th} and an arginine (R) at 591^{st} position which is known as SR polymorphism. The S590/R591provides mammalian adaptation in the absence of mammalian signatures at either 627^{th} or 701^{st} amino acid position (Mehle and Doudna, 2009).

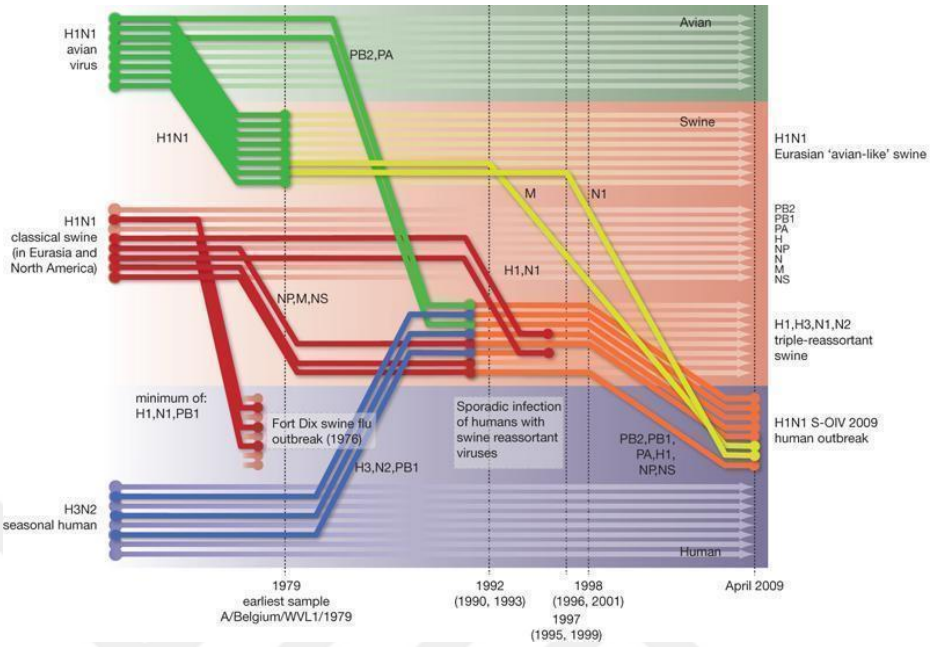


Figure 9. Reassortment events that gave rise to the emergence of 2009 Swine Flu virus. M and NA genes of the 2009 pandemic virus originated from Eurasian avian-like swine viruses. Origin of the other five segments was a triple reassortant virus which acquired its genes from human, swine and avian viruses (Smith *et al.*, 2009).

2.2.9. LPAI human infections

Apart from contribution to the emergence of pandemics, low pathogenic avian IAVs cause sporadic infections and outbreaks in humans. Although these infections generally require contact with infected birds, establishment of efficient human to human transmission is a major concern.

In 2013, a novel LPAI H7N9 virus emerged and infected more than 400 people and resulted in 60 deaths in one year (Zhang W. *et al.*, 2014). The case number exceeded 1500 until 2017 with a mortality rate of 38% (Guan *et al.*, 2018). Phylogenetic analyses of the viral gene segments indicated that the emergence of the virus was due to multiple reassortment events (Figure 10). Genomic analyses showed that the virus obtained its HA gene from a duck origin H7N3 virus, NA gene from a H7N9 virus circulating in wild birds and six other genes from H9N2 virus that established and has been circulating in poultry for years (Liu *et al.*, 2013). HA and PB2 proteins of the virus contained Q226L and E627K amino acid substitutions, respectively, which are markers for mammalian adaptation.

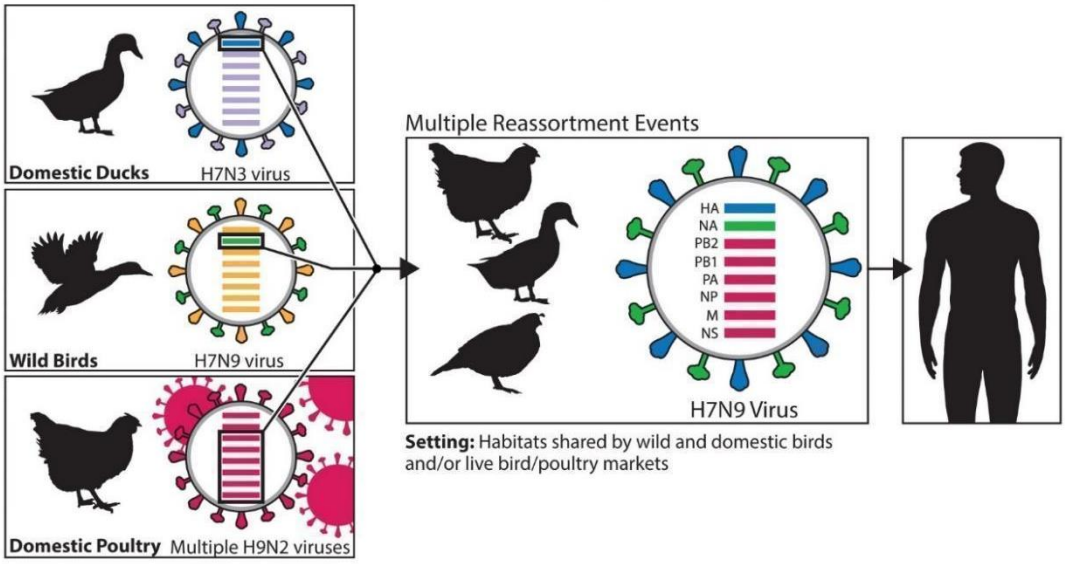


Figure 10. Formation of LPAI H7N9 virus via reassortment. The H7N9 virus that emerged in 2013 was a result of multiple reassortment events among viruses from wild and domestic birds. The virus acquired its HA gene from domestic duck, NA gene from wild birds and six internal genes from poultry (CDC, 2020a).

In another case, an H10N8 virus was isolated from patients admitted to hospital with respiratory symptoms in China in 2013. Analyses on the viral genome indicated that the virus obtained its six internal genes from H9N2 viruses which are established in poultry. HA and NA genes of the virus originated from wild aquatic birds. The HA protein of the virus contained Q226L and G228S amino acid substitutions that render shift in SA receptor specificity from avian to human. Another mammalian adaptation marker detected in the viral genome was E627K amino acid change in PB2 gene (Zhang T *et al.*, 2014, Chen *et al.*, 2014).

In 2013, an H6N1 virus was isolated from a patient. The virus obtained its PB2, PA and M genes from H5N2 viruses, PB1, HA, NP, NA and NS genes from H6N1 viruses circulating in poultry. The HA protein of the virus contained a serine amino acid at 228th position which indicates greater affinity for mammalian type SA receptor. HA protein also harbored P200L and A301T amino acid changes which are close to the receptor binding pocket. Moreover, NA protein had 12 amino acid long deletion at the stalk region of the protein which also correlated with human adaptation (Yuan *et al.*, 2013). Antibodies for H6 viruses were also detected in 63 people,

who are in close contact with either poultry or wild birds, in a serological surveillance study (Xin *et al.*, 2015). Another interesting example of IAV zoonosis was detected in 2016 in New York, US. A low pathogenic H7N2 virus was detected in a cat that transmitted the virus to other cats in the shelter. Then the outbreak spread to several other shelters where one of the caretakers was also infected with the same virus. Genetic analyses on the virus isolated from cats and the caretaker indicated that the virus was closely related to avian origin H7N2 viruses (Marinova-Petkova *et al.*, 2017).

As it can be seen, apart from pandemics, LPAIs continue to cause sporadic infections in humans. Moreover, these viruses possess a series of mammalian adaptation markers which makes each one of them a candidate for the next IAV pandemic.

2.3. Gediz Delta

Turkey is geographically important in terms of wild aquatic bird migration, as branches of two of the major flyways, Mediterranean/Black Sea and West Asian/East African Flyways, cross over Turkey (Figure 11). These flyways also intersect with several other major flyways where a great variety of aquatic birds from different geographical origins can come together for feeding, breeding, and/or resting during their migration periods. Since these birds may carry different subtypes and/or strains of IAVs, such meeting locations could be hotspots for reassortment events and emergence of novel IAVs.

Gediz Delta is one of the most important wetlands of Turkey, which has been protected by the Ramsar Treaty since 1994. The Delta is visited by 60000-90000 aquatic birds every year and 295 different species of wild aquatic birds have been identified in the area so far (KOSKS, 2012, 2013, 2020). The Gediz Delta also has a rich habitat that provides shelter and feeding opportunities for many mammals. The mammalian species detected in the Delta are weasels, horses, wild boars, golden jackal, red fox, wildcats, jungle cats which are previously shown to be susceptible for IAV infection (Reperant *et al.*, 2008; Shimoda *et al.*, 2017; Mucha *et al*., 2018; Marinova-Petkova *et al*., 2017, Soilemetzidou *et al.*, 2018). Species richness in the Delta provides IAVs the perfect ecosystem to reassort and/or to adapt to mammals. Izmir Bird Paradise National Park is also located in the Gediz Delta region. Thus, the area is also visited by humans for bird watching, scientific research or sportive activities such as cycling and jogging which

increases the risk of avian IAVs to transmit to humans. Therefore, a surveillance study to detect IAVs carried by wild aquatic birds in the Delta is important to identify IAVs that confer potential for zoonosis.

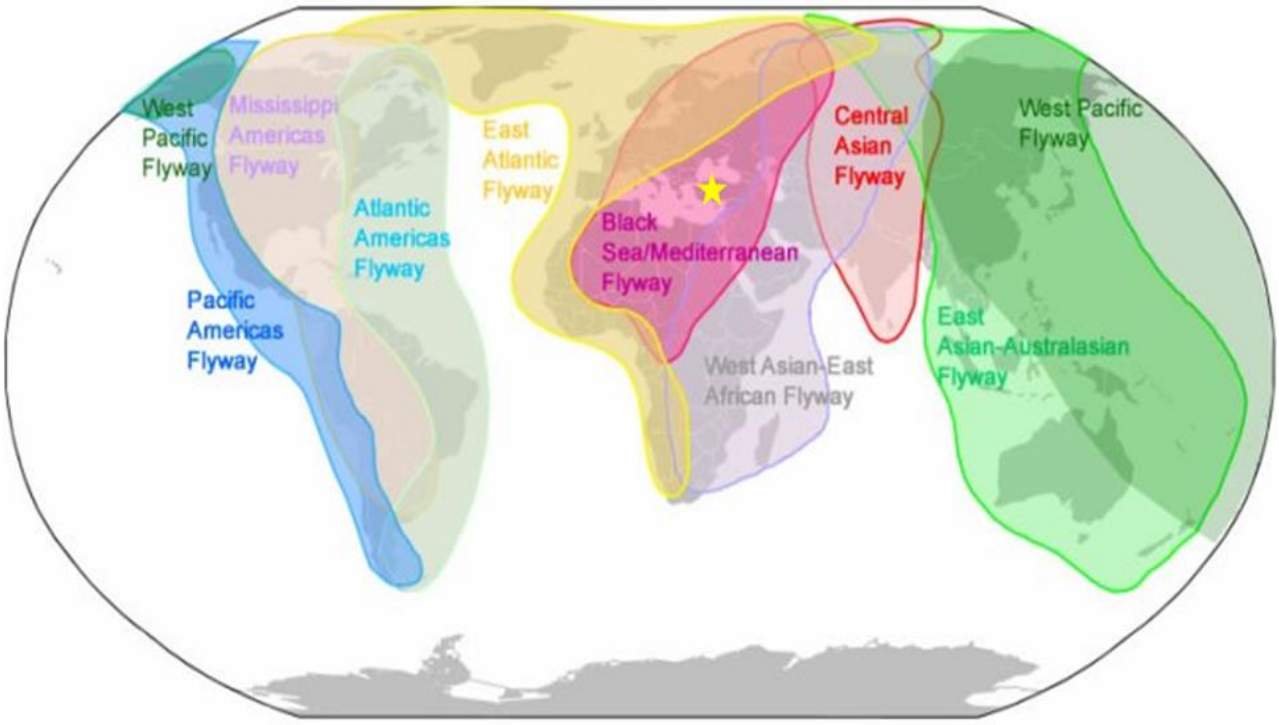


Figure 11. Major flyways around the world. Black Sea/Mediterranean Flyway is indicated with purple, West Asian/East African Flyway is indicated with grey. Turkey is designated with a yellow star (https://wysinfo.com/migratory-birds-without-boundaries/).

3. MATERIALS AND METHODS

3.1 Type of the Study

This thesis study is a surveillance study that includes the detection of the prevalence of avian IAVs residing in Izmir Bird Paradise located in Gediz Delta, the isolation of avian IAVs and molecular and phylogenetic characterization of isolated viruses.

3.2 Time and Place of the Study

Fresh fecal droppings were collected from Izmir Bird Paradise, Gediz Delta between December, 2017 and June, 2018. All experimental procedures were carried out at the Emerging Viral Diseases Laboratory (Koçer Lab) at Izmir Biomedicine and Genome Center.

3.3 Materials of the Study

Fresh fecal samples of wild aquatic birds were collected from Izmir Bird Paradise, Gediz Delta. Embryonated chicken eggs and chicken red blood cells were kindly provided by "EGE-TAV A.Ş.".

3.4 Variables of the Study

Independent variables of this study are; sample collection times and genes at which molecular markers were located.

Dependent variables of the study are; prevalence of IAVs based on sampling time and number of markers amino acid identified in each gene.

3.5 Tools for Data Collection

3.5.1. Materials used in the research

Table 1. List of plastic and glass consumables

Material	Brand	Catalog Number
1.5ml tubes	Greiner	616201
0.2ml (PCR) tubes	Thermo Fisher	AB-0620
15ml Centrifuge Tubes	Sarstedt	62.554502-500
50ml Centrifuge Tubes	Sarstedt	62.547254-300
2ml Nunc Cryotubes (internal thread)	Thermo Fisher	377267
Cotton Swabs	LP Italiana	L112498
2ml Cryotubes (external thread)	Sarstedt	72379992
U-Bottom 96-well plates	LP Italiana	650101

Plate sealing tapes	Isolab	I.124.01.001
145/225mm glass pasteur pipettes	Isolab	I.084.01.001/I.084.01.002
Rubber pump for pasteur pipettes	Isolab	084.03.001
10µl micropipette tips	Thermo Fisher	4130N00
200µl micropipette tips	Thermo Fisher	943300120
1000µl micropipette tips	Thermo Fisher	94300220
10µl/20µl/200µl/1000µl filtered micropipette tips	Neptune	BIO-BT10/20/200/1000
1ml/2ml/5ml/10ml/25ml/50ml Serological Pipets	SPL	I.083.03.001/002/005/010/ 025/050
1L/500ml/250ml/100ml glass bottles	Isolab	I.061.01.901/500/250/100
Graduated Cylinder 1L/500ml/250ml/100ml/50ml	Isolab	S.015.01.901/500/250/100/50
Gloves	Myglove	MYHN2L

Table 2. List of kits, reagents, solutions, chemicals, and antibiotics

Material	**Brand**	**Catalog Number**
Viral RNA Mini Kit (AVL buffer, Carrier RNA, AW1 buffer, AW2 buffer, AVE buffer, spin columns, collection tubes)	Qiagen	52904
RNeasy Mini Kit (RLT buffer, RW11 buffer, RPE buffer, nuclease free water, spin columns, collection tubes)	Qiagen	74104
B-Merchaptoethanol	Merc	8.05740.0250
QIAquick PCR Purification Kit (Buffer PB, buffer PE, buffer EB, pH indicator, spin columns, collection tubes)	Qiagen	28106

QIAquick Gel Extraction Kit (Buffer QG, buffer PE, buffer EB, pH indicator, spin columns, collection tubes)	Qiagen	28704
One-Step RT-PCR Kit (OneStep RT-PCR Buffer-5x, OneStep RT-PCR Enzyme Mix, dNTP mix, Nuclease free water)	Qiagen	210212
2X PCR master mix	Fermantas	K0171
PBS tablets	Sigma	P4417
TAE buffer 50X	BioShop	TAE222.1
Agarose	BioMax	HS-8000
Gel Loading Dye 6X	Fermantas	R0611
Safe View	Abm	G108
100bp DNA ladder	NEB	N3231S
Ethanol (96%)	Alkomed	-
Microchem	NCL	0255-29
VirkonS	Antek	VirkonS
Glycerol	Sigma	G2025
Penicillin G	Sigma	P3032-10MU
Streptomycin	Sigma	S9137
Gentamycin sulfate	Sigma	G1914
Nystatin	Sigma	N6261-500KU
Polymyxin B	Sigma	P4932-1MU
Paraffin Wax	Demirler Medikal	-

Table 3. List of equipment

Equipment	Brand	Model
Binoculars	Canon	CANON 12x36 IS III
-86^{o}C freezer	Eppendorf	HEF U410
-20^{o}C freezer	Bosch	GSN51AW30
+4^{o}C fridge	Bosch	KSV36AI31
Thermal Cycler	Thermo Scientific ™	SimpliAmp
Gel Electrophoresis system	Thermo Scientific ™	Owl™ EasyCast™ B1A Mini Gel Electrophoresis Systems
Gel Electrophoresis system Power Supply	Bio-Rad	PowerPac™
Gel imaging device	Bio-Rad	Molecular Imager Gel Doc XR System (170-8170)
Microwave	BEKO	MWB 200 EXN
Microcentrifuge	Thermo Scientific ™	MicroCL 17R
Fume Hood	Kötterman	2-401
Egg Incubator	Brinsea	Ova-Easy 380 Advance EX
Carving tool	Dremel	Micro 8050
Biological Safety Cabinets	Thermo Scientific ™	TS Safe 2020 Class IIA2 BSC
Waterbath	Nuve	NB 9
Microbiological Incubator	Thermo Scientific ™	Heraus IGS60
Soldering Iron	Proskit	8PK-S112B
Magnetic Stirrer	Thermo Scientific ™	Cimarec SP88857105
Vortex Mixer	Thermo Scientific ™	LP Vortex Mixer
Balance	Sartorius	Entris 822-1S
Mini Centrifuge	Thermo Scientific ™	MySpin 6 Sprout-mini Centrifuge

Micropipette Set (2µl-20µl; 20µl-200µl; 100-1000µl)	Gilson	NEO Starter Pipet Kit
Micropipette Set (2µl-20µl; 20µl-200µl; 100-1000µl)	Eppendorf	Research® plus
Multichannel pipette (30µl-300µl)	Eppendorf	Research® plus
Micropipette (0.5µl-10µl)	Eppendorf	Research® plus

Table 4. List of primers used for amplification

Primer[a, b]	**Sequence (5' to 3')**	**Melting Temperature (T_m)**
PB2-1F	TATTGGTCTCAGGGAGCGAAAGCAGGTC	68
PB2-2341R	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	65
PB1-1F	TATTCGTCTCAGGGAGCGAAAGCAGGCA	68
PB1-2341R	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT	63
PA-1F	TATTCGTCTCAGGGAGCGAAAGCAGGTAC	68
PA-2233R	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT	63
PA-1718R	ATATCGTCTCGTATTACATACAGGAACATGGGCCT	68
PA-1102F	TATTCGTCTCAGGGAATGAAGAAAACAAGCCAGTT	67
HA-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	68
HA-1770R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	65
HA-1144F	GGAATGATAGATGGNTGGTAYGG	61
H5-918F[c]	CCARTRGGKGCKATAAAYTC	56
H5-1166R[c]	GTCTGCAGCRTAYCCACTYC	60
H7-950F[d]	GCCCTCGRTATGTCARACA	57
H7-1143R[d]	TTTGTARTCAGCTGCAGTYC	55

NP-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTA	67
NP1565R	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTTC	65
NA-1F	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	67
NA-1413R	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	64
M-1F	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	67
M-1027R	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	64
M-30F[c]	ATGAGYCTTYTAACCGAGGTCGAAACG	65
M-264R[c]	TGGACAAANCGTCTACGCTGCAG	63
NS-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	68
NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	65

[a]F: forward, R: reverse
[b]Numbers indicate the nucleotide position on each gene segment
[c]WHO, 2012
[d]Leion *et al.*, 2011
Sequences of the rest of the primers were kindly provided by the Influenza Surveillance Laboratory, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

Table 5. List of primers used for sequencing

Primer [a, b]	**Sequence (5' to 3')**	**Melting Temperature (T_m)**
PB2-1F	TATTGGTCTCAGGGAGCGAAAGCAGGTC	68
PB2-2341R	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	65
PB2-1252R	ATATGGTCTCGTATTAATCTTCTTGTGAGAACACCAT	66
PB2-949F	TATTGGTCTCAGGGAGTGGATATATGCAAGGCAGCAATGG	73
PB2-514F	ATCATGGAGGTTGTTTTCCCHAA	58
PB2-1475F	TGGGNGTRGATGARTAYTC	55
PB2-1948F	AGRGGHTCDGGRATGAGAATAC	60

PB2-1962R	ATTCCKGAGCCTCTCACATTCACAG	64
PB2-555R	TTCATTTGGGAAAACGACCTCCATG	61
PB2-765R	TGGAGTGTACATTTGTTCCCAGCA	61
PB1-1F	TATTCGTCTCAGGGAGCGAAAGCAGGCA	68
PB1-2341R	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT	63
PB1-1225R	TATTCGTCTCAGCTGTACCATCTATTAATAGAGGCCTTAT	68
PB1-683F	TATTCGTCTCAGGGAGAGCACTGACATTGAACACAATG	71
PB1-612R	CATTTTCTTGGTCATGTTG	50
PB1-1944F	AACAATGCTGTGGTGATGCC	57
PB1-1964R	GGCATTACCACAGCATTGTT	55
PB1-1224F	ACAGCCTCATTGAGTCCTGGAATGATGATG	67
PA-1F	TATTCGTCTCAGGGAGCGAAAGCAGGTAC	68
PA-2233R	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT	63
PA-1718R	ATATCGTCTCGTATTACATACAGGAACATGGGCCT	68
PA-1102F	TATTCGTCTCAGGGAATGAAGAAAACAAGCCAGTT	67
PA-587F	GGGAYTCCTTTCGTCAGTCCG	63
PA-607R	CGGATTGACGAAAGGAGTCCC	62
PA-1109R	TTYTTCATRTTYTTNGTYYTTGG	64
PA-1543F	GGARTTYTCYCTYACWGAYCC	59
PA-1408R	ATTCAACAGGGCTGTGTTTATGTACACTCCC	67
PA-915F	GCTGTATGATGCKATCAAGTGYAT	59
PA-368F	GAGAACCGATTCATYGAGATTGG	60
PA-2050F	TCAGGCTCTTAGGGACAATCTKGAGCCTG	69
HA-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	68
HA-1770R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	65

HA-1144F	GGAATGATAGATGGNTGGTAYGG	61
H6-450F	TAGATACTAATAGCGGAGTTACGA	58
H6-476-R	TCGTAACTCCGCTATTAGTATCTA	58
H6-1050F	TGAGAAATGTTCCACAGGCAGAAAC	61
H6-1074R	GTTTCTGCCTGTGGAACATTTCTCA	61
H6-1376F	AATGCTGAACTTCTGGTTCTGCTGG	63
H6-1389R	CCAGCAGAACCAGAAGTTCAGCATT	63
H6-444R[c]	TGTCAACTCCTGTCCATGTGC	60
H6-1026R[c]	TCCTCAGTCCAGTTGCAAGC	59
NP-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTA	67
NP-1565R	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTTC	65
NP-517R	GGAATGGAYCCCAGGATGTGCTC	65
NP-548R	TGCATTAGAGAGCACATTCTGGGGTCC	66
NP-1031F	TGTGGATGGCATGCMATTCTGCHGC	66
NP-264R	CAAATGCWGCAGAGTKGCATGCCATCC	67
NA-1F	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	67
NA-1413R	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	64
N2-500F	TTATCGGACCCTTTTGATGAATG	57
N2-515R	AAAAGGGTCCGATGAGGGCTTCTATCAT	65
N2-1085F	GAGTGAAAGGCTGGGCCTTTGATGA	65
N2-1086R	TCATCAAAGGCCCAGCCTTTTACTC	63
M-1F	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	67
M-1027R	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	64
M-339F	GGGAGATAACATTCCATGGG	57
M-787R	TTGAATCGTTGCATCTGTAC	53

M-30F	ATGAGYCTTYTAACCGAGGTCGAAACG	65
M-264R	TGGACAAANCGTCTACGCTGCAG	63
NS-1F	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	68
NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	65
NS-420F	ACTTCAGTGTGATTTTTGACCGG	59
NS-446R	CCGGTCAAAAATCACACTGAA	56

[a]F: forward, R: reverse

[b]Numbers indicate the nucleotide position on each gene segment

[c]Primers designed in this study. Sequences of the rest of the primers were kindly provided by the Influenza Surveillance Laboratory, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

3.5.2. Experimental methods

3.5.2.1. Sample collection

Fresh fecal droppings of wild aquatic birds were collected at Izmir Bird Paradise, Gediz Delta. Before sampling, areas where wild birds clustered were observed using binoculars. For sampling, the areas where the number and diversity of birds seemed adequate were selected. Sampling was carried out as follows: First, fresh looking moisty bird feces were collected from the ground by swabbing a cotton swab onto the feces. The cotton tip was dipped into the cryogenic tube containing 1ml viral transport media (Table 6) and the tip of the swab was cut around 1cm above. Samples were stored in a cooler box during the field study and were frozen using dry ice before transferring to the -86^{o}C freezer for storage.

3.5.2.2. Viral RNA Isolation

Before viral RNA extraction, samples were taken from the -86^{o}C freezer and transferred to the virus culture laboratory which is assigned only for influenza research. Samples were thawed in a water bath adjusted to 37^{o}C in the virus culture laboratory. Then, the tubes were transferred to Class II A2 biosafety cabinets to perform RNA isolation. Based on availability, two different RNA isolation kits were used throughout the study.

Table 6. Components of the viral transport media

Material	Amount (in 1L)
Glycerol (Autoclaved)	500ml
PBS (Autoclaved)	Up to 1L
Penicillin	1x 10^6 U/L
Streptomycin	200mg/L
Gentamicin Sulfate	150mg/L
Polymyxin B	5000 U/L
Nystatin[a]	25000 U/L

[a]Stocks of all antibiotics were prepared in sterile ddH_20 and all were filter-sterilized except Nystatin. All aliquots were stored at -20℃.

i. QIAGEN, QIAamp Viral RNA Mini Kit

Approximately 200µl of each sample was withdrawn using 1cc tuberculin syringe (26 gauge, 1.5 inch). To withdraw the sample, the needle of the syringe was stabbed into the cotton tip of the swab which was placed in the tube at the sample collection. The sample was withdrawn into the syringe by filtering the liquid through the cotton tip of the swab. Then, the sample was transferred into a sterile 1.5ml centrifuge tube and the tube was spun using tabletop mini centrifuge for ~1 minute. This step was performed in order to precipitate large particles that may clog the column filters used in viral RNA isolation procedure.

Per sample, 560µl of AVL buffer was mixed with 5.6µl carrier RNA in sterile 1.5ml tubes. Then, 140µl of each sample was transferred into the AVL buffer/carrier RNA mixture and mixed using a vortex for 30 seconds to break the viral envelope. Then the mixture was incubated for 10 minutes at room temperature. After incubation, 560µl 96% ethanol was added to the mixture and solution was mixed by pulse vortexing for 15 seconds. 630µl of the mixture was transferred into the spin columns and centrifuged in a microcentrifuge at 8000 rpm for 1 minute. The collection tubes containing the flow-through were discarded and replaced with a new one. Remaining sample mixes were transferred into the columns and centrifuged at 8000 rpm for 1 minute. Then, 500µl AW1 buffer (ethanol added) was added into the columns to wash and

centrifuged at 8000 rpm for 1 minute. The collection tubes were replaced with new ones and 500µl AW2 buffer (ethanol added) was added into the columns for the second wash step and centrifuged for 3 minutes at 13300 rpm. Flow-throughs were discarded, and the columns were centrifuged for an additional 1 minute at 13300 rpm to remove residual ethanol which may interfere with the downstream applications such as RT-PCR. Then, the columns were placed into sterile 1.5ml sterile centrifuge tubes. 60µl AVE buffer was gently added onto the column filter and incubated at room temperature for 3 minutes. The columns were centrifuged at 13300 rpm for 1 minute. Extracted viral RNAs were stored at -20^{o}C freezer until further analysis.

ii. QIAGEN, RNeasy Mini Kit

For each sample, 200µl RLT buffer was mixed with 4µl beta-mercaptoethanol in a sterile 1.5ml centrifuge tube. Approximately 250µl of each sample was withdrawn using a 1cc tuberculin syringe by filtering the sample through the cotton tip of the swab and transferred into a sterile 1.5ml tube. Then the withdrawn samples were spun for ~1 minute using a tabletop mini centrifuge to precipitate particles which may clog the column filter. 200µl of the samples were mixed with the RLT buffer/beta-mercaptoethanol mix. The solution, then, was mixed using a vortex for 1 minute. After that 400µl of 70% ethanol was added into each tube and mixed by vortexing. After incubating at room temperature for 10 minutes, 400µl of mixes were transferred into columns and centrifuged at 13300 rpm for 15 seconds. Flow-throughs of each sample were discarded and remaining sample mixes were transferred into columns. After centrifugation at 13300 rpm for 15 seconds, the flow-throughs were discarded. Then, 700µl RW1 buffer was added into columns and centrifuged at 13300 rpm for 15 seconds. Flow-throughs were discarded and a 500µl RPE buffer was added into each column. The columns were centrifuged at 13300 rpm for 2 minutes. In order to remove residual ethanol, empty columns were centrifuged at 13300 rpm for 1 min. Then, each column was placed into a sterile 1.5ml centrifuge tube. 50µl nuclease-free distilled water was gently added directly onto the membrane of each column and incubated at room temperature for 10 minutes. Tubes were centrifuged at 13300 rpm for 1 minute to elute RNAs attached to the column membrane. Columns were discarded and eluted RNAs were stored at -20^{o}C until further analyses.

3.5.2.3. One-Step RT-PCR

10µl RT-PCR reactions were set for the initial detection of IAV-positive samples based on the amplification of a small region (234bp between 30-264 nt) of M gene (M-partial) and for the identification of H5/H7 positive samples, if any. Then, M-partial amplifications were performed in 30µl reactions for RT-PCR positive samples to send for confirmation by sequencing. After virus isolation, 50µl reactions were set to amplify all gene segments of the isolated virus, which were sent for sequencing to perform genome analyses.

For all reactions, a reaction mix was prepared for samples in a sterile 1.5ml centrifuge tube. The amounts of the components are multiplied with the number of samples including controls. The final concentrations of each component in a reaction mixture was as follows: 1X of 5X reaction buffer mix, 0.6µM of each forward and reverse primers, 400µM of each dNTPs, 0.04µl/µl of enzyme mix (Table 7).

Table 7. Content of reaction mixture

	Final concentrations in each reaction	10µl reaction[b]	30µl reaction[b]	50µl reaction[c]
Nuclease free dH_2O	up to reaction volume	4µl	12µl	30µl
5X Reaction Buffer	1X	2µl	6µl	10µl
Forward Primer[a]	0.2µM-0.6µM	0.6µl	1.8µl	1µl[c]
Reverse Primer[a]	0.2µM-0.6µM	0.6µl	1.8µl	1µl[c]
dNTP Mix	400µM each	0.4µl	1.2µl	2µl
Enzyme Mix	0.04µl/µl	0.4µl	1.2µl	2µl
Template	100 ng-1µg	2µl	6µl	4µl[c]

[a]Gene specific forward and reverse primers were used for each segment.

[b]10µl reactions were set for the detection of IAV positive samples; 30µl samples were set for sequencing to confirm the results.

[c]50µl reactions were set for the amplification of the genes of the isolated virus, thus the required amount of primers and template were decreased.

The reaction mix was distributed to 0.2ml PCR tubes and template RNAs (100ng-1µg) were added into corresponding tubes. Reaction mixes were mixed by tapping the tube several times. The PCR tubes were spun for 3-4 seconds to let any droplets down into the mixture. PCR tubes were placed into the thermal cycler and reaction conditions were set (Table 8). After the reaction was over, PCR tubes were removed from the thermal cycler and results were checked by agarose gel electrophoresis or transferred into the +4oC fridge to be analyzed later.

Table 8. RT-PCR reaction conditions

Step	Temperature	Time	Cycle number
Reverse transcription	50°C	1 hour	1
Initial denaturation	95°C	15 minutes	1
Denaturation	95°C	1 minute	40
Annealing[a]	56-60°C	1 minute	
Extension[b]	72°C	2-3 minutes	
Final extension	72°C	10 minutes	1

[a]Annealing temperature was set to 56°C for M, NS, NP, HA, HA2, NA; 60°C for polymerase genes.
[b]Extension time was set to 2 minutes for M, NS, NP, HA, HA2, NA; 3 minutes for polymerase genes.

3.5.2.4. Agarose gel electrophoresis

1g agarose powder was weighed using a precision scale and transferred into a 250ml glass bottle. 100ml 1X TAE buffer was measured using a graduated cylinder and poured into the glass bottle containing agarose powder to prepare 1% agarose gel. The suspension was mixed by shaking the bottle gently. Then the mixture was heated in the microwave oven for ~2 minutes. While the agarose solution was being heated, the gel tray was placed into the gel caster and a comb was placed on the tray. After the gel mixture was heated, the glass bottle was removed from the microwave oven and cooled down. A 5µl safeview classic (0.05µl/ml) was added into 100ml gel mix and the solution was mixed by shaking the bottle. Then, the gel mix was poured into the plastic tray and let to cool down and solidify.

After the gel solidified, the comb was removed without damaging the gel. Then, the gel was transferred into the gel tank containing 1X TAE buffer. A piece of parafilm was cut and for each sample, including positive and negative controls, 0.6µl 6X loading dye per sample was placed on the paraffin surface of parafilm as separate drops. Then, 3µl of PCR products were mixed with loading dye on the parafilm by pipetting. Each sample mix was transferred into wells of the agarose gel. Power supply was set to 90V and 45 minutes. Then the lid of the gel tank was closed, and gel electrophoresis was started. After 45 minutes was over, agarose gel was gently removed from the tank and transferred to the gel imaging device to visualize the gel.

3.5.2.5. PCR Purification

PCR purification was performed using QIAquick PCR Purification Kit for the amplicons yielded a single band in agarose gel electrophoresis. One volume of each amplicon was combined with five volumes of buffer PB and mixed by inverting the tube several times. Mixtures were transferred into columns and centrifuged at 13000 rpm for 1 minute. Then, the flow-throughs were discarded and 750µl buffer PE (ethanol added) was added to each column. Columns were centrifuged at 13000 rpm for 1 minute. In order to remove residual ethanol, empty columns were centrifuged at 13000 rpm for 1 minute and the columns were placed into sterile 1.5ml centrifuge tubes. 30µl buffer EB was gently added directly onto the column membrane and columns were incubated for 3 minutes at room temperature. Then, the columns were centrifuged at 13000 rpm for 1 minute to elute the amplicons. Purified amplicons were stored at -20°C for further analyses.

3.5.2.6. Gel extraction purification

Gel extraction purification was performed using QIAquick Gel Extraction Kit for the amplicons yielded multiple bands in agarose gel electrophoresis. First RT-PCR products were loaded on an 1% agarose gel and were run for 45 to 60 minutes at 90V. The agarose gel was placed on the UV-transilluminator. The bands of interest were visualized on the gel with UV light and excised from the gel using a sterile scalpel. Gel pieces were transferred into sterile 1.5ml centrifuge tubes and weighed. Then, three volumes of QG buffer were added in each tube and mixtures were incubated in a 37^{o}C water bath up to 10 minutes till gel pieces were dissolved completely. The mixtures were transferred into columns and centrifuged at 13000 rpm for 1 minute. The flow-throughs were discarded and a 500µl QG buffer was added into columns.

Columns were centrifuged at 13000 rpm for 1 minute and flow-throughs were discarded. After that, 750µl PE buffer was added to columns and centrifuged at 13000 rpm for 1 minutes. Flow-throughs were discarded, and the columns were centrifuged at 13000 rpm for an additional 1 minute to remove the residual ethanol. Then, the columns were placed into sterile 1.5ml centrifuge tubes and 50µl buffer EB was gently added directly to the column filters. After 2 minutes incubation at room temperature, tubes were centrifuged at 13000 rpm for 1 minute to elute the amplicons. Purified amplicons were stored at -20^{o}C until further analyses.

3.5.2.7. Virus propagation and isolation

Avian IAVs attach to the host cells through α-2,3 linked SA receptors which is also the major SA receptor type in the allantoic fluid of embryonated chicken eggs (ECE) (Ito *et al.*, 1997). For that reason, ECEs are widely used in IAV isolation and propagation. Thus, in this thesis study, ECEs were used to propagate and isolate viruses from the fecal samples that were found to be virus-positive based on the amplification of the matrix gene of IAVs.

i. Inoculation of RT-PCR positive samples into ECEs

ECEs from IAV-negative flocks were kindly provided by “EGE-TAV A.Ş. " on day 0, the day at which the eggs were laid. Damaged eggs were discarded, and the rest of the eggs were placed into the egg incubator. The incubator was set to 37.5^{o}C, 50% humidity and rack rotation in every 45 minutes. ECEs were checked at 4th, 7th, and 9th days for embryonic development by putting light source onto each egg in the dark. This procedure is called “candling”. Eggs without embryonic development were discarded. On day 9, the location of the air sac of each egg was determined and designated by drawing a line on the air sac. The same day PBS-antibiotic solution (2000U/ml penicillin, 400U/ml streptomycin, 200U/ml polymyxin B, 0.05mg gentamicin) was prepared and 300µl of mixture, for each sample, was withdrawn into 1cc tuberculin syringes and stored in 4^{o}C.

On day 10, ECEs were taken out from the egg incubator and observed by candling before inoculation to make sure all embryos were alive. ECEs were labeled with the names of the samples and surface-sterilized by spraying with 70% ethanol. Using a carving tool, each egg was drilled on the air sac line, forming a small hole on the eggshell without disrupting the membrane. After spraying with 70% ethanol, ECEs were taken into the biosafety cabinet.

In order to inoculate samples into ECEs, 300µl of each sample was withdrawn into the sterile syringe, which contained 300µl PBS-antibiotic mix, by filtering the sample through the cotton tip of the swab, as described in section "3.5.2.2. Viral RNA extraction". Then, the solution was mixed by taking a small bubble into the syringe and inverting the syringe several times. Then, 200µl sample/PBS-antibiotics mixture were inoculated into each ECE by inserting the syringe through the hole on the eggshell. Inoculation of each sample was carried out in triplicates. After that, inoculated ECEs were sprayed with 70% ethanol and taken out of the biosafety cabinet. Punctures on the eggs were sealed by dropping one or two drops of melted paraffin wax using a glass pasteur pipette and the paraffin wax was let solidify. After inoculation, ECEs were put into the microbiological incubator with natural convection heat transfer which was set to 35^{o}C. Inoculated ECEs were incubated for 3 days and status of embryos were checked every day. At the end of 3rd day, eggs were placed in the +4^{o}C fridge to euthanize the embryo when they are 13 days old.

ii. Preparation of chicken red blood cells (CRBCs)

Chicken blood was kindly provided by "EGE-TAV A.Ş.". The blood was transferred into a 15ml tube and centrifuged at 1200 rpm for 10 minutes. Supernatant was taken out using a serological pipet and discarded. After that, serial wash steps with PBS were performed. Each step involved the addition of PBS onto the pellet up to 10ml and mixing the suspension by gently inverting the tube. After each PBS addition, tubes were centrifuged at 1200 rpm (10 minutes), 3000 rpm (15 minutes), 4400 rpm (20 minutes), respectively, and supernatants were discarded. After the last centrifuge step, packed CRBCs were stored in +4^{o}C fridge.

To prepare the working solution of CRBC (0.5%), 50ml PBS was added into a 50ml tube and ~0.1ml PBS from the tube was withdrawn into a 1ml serological pipette. Then 0.25ml packed CRBC was withdrawn into the same pipette. In this stage, PBS was withdrawn since the packed CRBC is viscose and needs the pressure applied by PBS to be discharged from the pipette. The CRBC/PBS in the serological pipette was transferred into the 50ml PBS, and the solution was mixed by inverting the tube.

iii. Hemagglutination Assay

Virus propagation in ECEs was checked using hemagglutination assay (HA assay) which takes advantage of the ability of influenza virus to agglutinate red blood cells. First, 50µl PBS was added into the wells in rows C, D, E and F. Then, ECEs were removed from the +4°C and taken into the biosafety cabinet. Paraffin seals on the punctures were reopened using soldering iron. From each ECE, approximately 200µl allantoic fluid was withdrawn using a glass pasteur pipette and transferred into designated well in row A or row H of U-bottom 96-well plate. Then, 50µl of allantoic fluids in row A were transferred into row B using a multichannel pipette. Then, 50µl allantoic fluid from the wells of row A was serially diluted in rows C and D (row B: undiluted, row C: 1/2 dilution, row D: 1/4 dilution). The same process to dilute the samples in row H was carried out for rows G (undiluted), F (1/2 dilution) and E (1/4 dilution) (Figure 12A). After that, 50µl 0.5% CRBC was added into each well. The plate was gently tapped several times from each side to mix the samples with CRBC. Then, the plates were covered with adhesive plate seal tapes and incubated at room temperature for 30 minutes. Hemagglutination activity was checked at the end of the incubation period. Positive samples form a homogeneous mixture as CRBCs are attached to the viruses. On the other hand, CRBCs precipitate and form a red dot at the bottom of the plate, if the sample is virus-negative (Figure 12B).

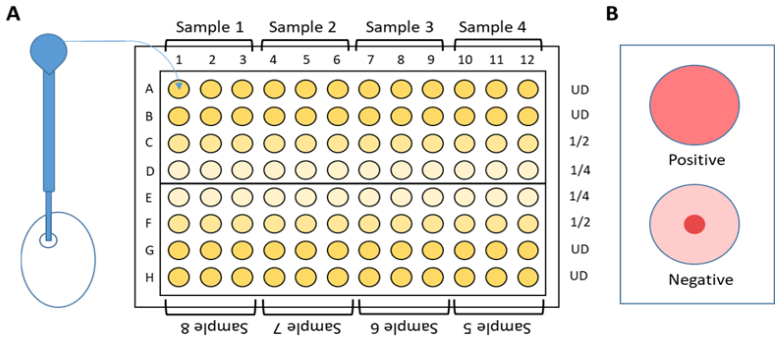


Figure 12. Diagram representing the plate organization in hemagglutination assay. Allantoic fluid samples from each egg were transferred into respective wells in row A and H, then serially diluted to row D and E. "UDs" in the right panel of the image stands for "undiluted" (A). After incubation with CRBCs, homogeneous mixture is observed for the positive samples, whereas a red dot is formed in negative samples (B).

iv. Harvesting allantoic fluid

After the hemagglutination test, the tops of the eggs were cracked open from the air sac region using sterile forceps. Using a sterile grooved spatula, embryos were gently pushed one side of the egg without disturbing the egg yolk. Then, a sterile serological pipette was put on the spatula and 5 ml allantoic fluid was withdrawn. Total of 15ml of allantoic fluid for each sample (5ml/egg) was collected in sterile 15ml tubes. After collection is finished, 5µl of each allantoic fluid sample was inoculated onto 5% sheep blood agar plates and incubated overnight at 37^{o}C to detect bacterial contamination, if there is any. If there was bacterial growth on the plates, allantoic fluids were filter-sterilized through 0.22nm polyethersulfone (PES) syringe filters and re-tested by hemagglutination assay to confirm virus presence. Because some bacteria are known to agglutinate red blood cells. Ten to 15 aliquots (1ml/tube) of positive samples were prepared and stored in -86°C. Two aliquots (1ml/tube) of negative samples were stored to be used in next passages in ECEs up to 3 passages. Each aliquot was taken in external threaded cryotubes and frozen quickly in dry ice to prevent decrease in virus titers in the samples. Upon freezing, aliquots were stored in -86^{o}C until further use.

3.5.2.8. Determination of Viral Titer

Titer of the isolated virus was determined as Hemagglutination titer (HA titer) and 50% egg infective dose (EID_{50}).

i. Determination of HA titer

In order to determine the HA titer of the virus, HA assay was performed as follows: 50µl PBS was added in all rows of the U-bottom 96-well plates. 50µl sample (allantoic fluid) was added in the first row and serially diluted by 2-fold to rows 2-12 (Figure 13). Then, 50µl 0.5% CRBC was added in each well, plate was mixed by gently tapping the plate from each side. Then the plate was covered with adhesive plate seal tapes and incubated at room temperature for 30 minutes. Hemagglutination activity was checked at the end of the incubation period. The titer was determined as the highest dilution at which hemagglutination activity was detected.

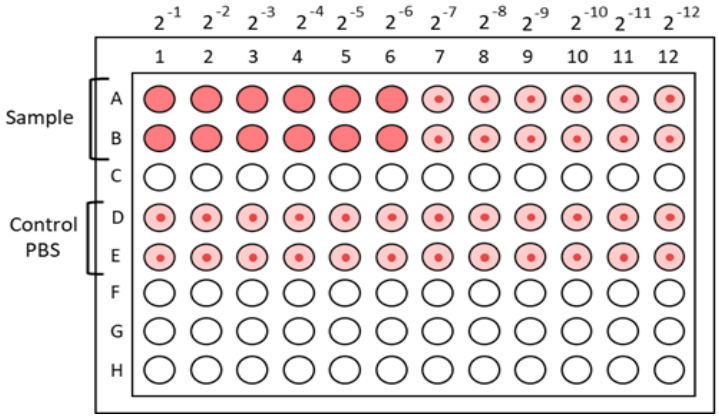


Figure 13. Diagram representing the plate organization in determination of HA titer. First, the sample was mixed with PBS in the first column then serially diluted by 2-fold to the column 12. PBS was used as a negative control. The HA titer was determined based on the highest dilution at which hemagglutination was detected (for example, HA titer shown in the diagram is 64 HA unit).

ii. EID_{50} measurement

EID_{50} value of the isolated virus were determined to identify the amount of the virus in the sample, which was able to infect 50% of the eggs. In order to measure the EID_{50} value of the virus, 10-fold dilutions were prepared (10^{-1}-10^{-10}) in PBS/antibiotics solution and 100µl of each dilution was injected into ECEs in triplicates as described in "3.5.2.7. Virus propagation and isolation". After 3 days of incubation, ECEs were incubated overnight at 4°C and HA assay was performed as described in "3.5.2.7.iii. Hemagglutination Assay". EID_{50} was calculated with the formula below (Reed and Muench, 1938):

$$\frac{\text{(\% of infected eggs immediately above 50\%) -50\%}}{\text{(\% of infected eggs immediately above 50\%) - (\% of infected eggs immediately below 50\%)}} = a$$

$$EID_{50} = \text{(dilution immediately above 50\% of eggs are infected)} \times 10^{a} \times 10$$

3.5.2.8. Sequencing

Purified amplicons were sent for sequencing via Sanger platform (Medsantek Ltd. Co.). The amount of amplicon sent for sequencing was determined based on the number of primers

used for sequencing (Table 9). Each amplicon was sent with 2µl of 10µM respective primer(s). Sequencing results were confirmed to belong to respective IAV segments using NCBI BLASTn program with the default options (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5.2.9. Sequence assembly and identification of marker amino acids

Sequencing reads, obtained from each primer for each gene, were assembled using DNASTAR Lasergene SeqMan PRO version 16 software (trial version in December 2019). Files with ".abi" extension for the reads obtained from each primer were uploaded to the program for each segment individually. Then, all reads for individual segments were assembled into one contig. BLAST analysis at nucleotide level was performed for each contig to confirm that the contigs belonged to the respective IAV segments.

Table 9. Number of primers and total amount of amplicons sent for sequencing

Gene	Number of primers[a]	Amount of amplicon sent for sequencing
M-Partial	1	4µl
HA-partial[b,c]	2	8µl
NA[b]	2	8µl
PB2	10	40µl
PB1	8	32µl
PA	12	48µl
HA	11	44µl
NP	6	24µl
M	6	24µl
NS	4	16µl

[a]Total number of primers used to sequence each partial or full-length amplicon. Each amplicon was sent with 2µl primer of respective segment.
[b]Sequencing performed for subtype identification.
[c]HA-partial: ~680bp portion of HA2 domain of HA gene.

Each confirmed contig was uploaded to the BioEdit program (Hall, 1999) and 5' ends of the sequences were trimmed till the start codon (ATG). Then nucleotide sequences were translated to amino acid sequences using the respective option in the program. Amino acids at each signature position of each gene segment were checked and compared with the lists of amino acids for mammalian adaptation, increased polymerase activity, pathogenicity or transmissibility that have been described in the literature so far (relevant literature are given in the Results and Discussion sections).

3.5.2.10. Phylogenetic analysis

Phylogenetic analyses were performed to identify the geographical origins of antigenic segments of the isolated virus. For this purpose, HA (H6) and NA (N2) sequences were downloaded from IRD (www.fludb.com).

H6 sequences from Europe and Africa were downloaded without any date restriction. However, H6 sequences from America and Asia were restricted to the years 2015-2020. Date restriction was applied to the American and Asian sequences due to the fact that the number of American and Asian H6 sequences were too high (n=1692) compared to other regions that could create a bias towards the results of the analysis. Thus, H6 sequences that are genetically similar to the HA gene of the virus isolated in this study were selected from more recent years. The number of H6 sequences used as representatives of different geographic origins are given in Table 10. The total of 242 representative H6 sequences were used to construct the phylogenetic relations.

Similarly, N2 sequences from Asia, Africa, Europe and North America that are genetically similar to the NA gene of the virus isolated in this study were downloaded for the years 2010-to-2019. Since the number of N2 sequences from Asia and North America was too high, they were individually grouped based on the year and redundant sequences from each year were removed using the Jalview program version 2.11.1.0 (98% cutoff) (Waterhouse *et al.*, 2009). The number of N2 sequences used as representatives of different geographic origins are given in Table 10. The total of 423 representative N2 sequences were used to construct the phylogenetic relations.

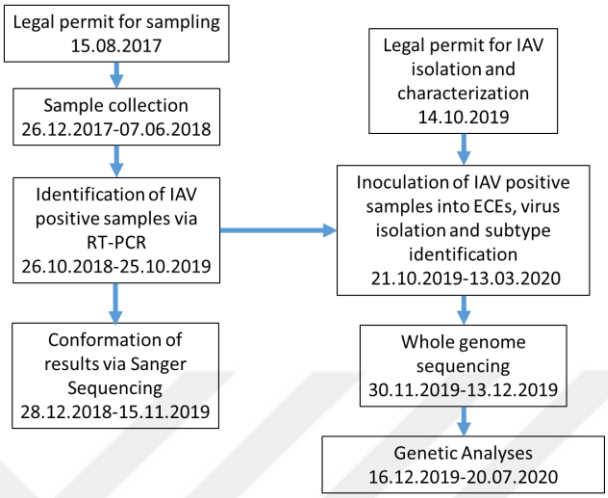
Table 10. Number of H6 and N2 sequences from respective years and locations

Years	Asia		Africa		Europe		America		Total	
	H6[a]	N2[b]	H6	N2[b]	H6	N2[b]	H6[a]	N2[b]	H6	N2
Before 2010	0	0	8	0	50	0	11[c]	0	69	0
2010	0	21	0	0	6	11	0	4	6	36
2011	0	23	0	1	19	8	0	9	19	41
2012	0	40	0	2	2	3	0	7	2	52
2013	0	60	0	2	1	3	0	10	1	75
2014	0	43	0	0	4	12	0	6	4	61
2015	27	33	0	1	2	2	29	5	58	41
2016	14	31	0	2	0	7	21	7	35	47
2017	14	26	0	0	0	1	13	14	27	41
2018	7	16	0	0	0	2	14	6	21	24
2019	0	5	0	0	0	0	0	0	0	5
2020[d]	0	0	0	0	0	0	0	0	0	0
Total	62	298	8	8	84	49	88	72	**242[e]**	**423[e]**

[a]H6 sequences between 2015-2020 from Asia and America were downloaded.
[b]N2 sequences between 2010-2020 from Asia and America was downloaded.
[c]H6 sequences before 2010 from America are from South America.
[d]There were no H6 or N2 sequences from 2020 in the database
[e]Total numbers of H6 and N2 sequences used in this study.

H6 and N2 sequences (n=242and n=423, respectively) were aligned using the BioEdit program (Hall, 1999). The alignment was saved as a Fasta file and uploaded to the MEGAX program to perform phylogenetic analyses (Kumar S. *et al.*, 2018). The evolutionary distances were calculated by Maximum Composite Likelihood method where both transition and transversions are included. Rates among sites were set as uniform and pattern among lineages were set as homogeneous. Gaps were treated with pairwise deletion and the phylogenetic trees of H6 and N2 were constructed using the Neighbor Joining method (Saitou and Nei, 1987; Tamura *et al.*, 2004). Bootstrap test was performed for each tree with 1000 replicates and the consensus trees were edited using FigTree program (version 1.4.4) (Rambaut, 2018).

3.6 Study Plan Calendar



3.7 Data Evaluation

Detection of prevalence: IAV prevalence was determined via RT-PCR and confirmation of the RT-PCR result via Sanger sequences. Prevalence depending on sampling times were plotted as a bar graph where confidence interval (CI) was assumed to be 95%.

Subtype determination: HA-partial and NA genes were sequenced using Sanger platform (Medsantek Ltd. Co.). HA and NA subtype were determined based on identities of the sequence reads in the database using NCBI BLASTn program.

Determination of virus titers: The titer of the virus was determined as HA titer and EID_{50}. HA titer determined based on the highest dilution at which hemagglutination was observed. EID_{50} was calculated with the formula given in the section "3.5.2.8. Determination of Viral Titer".

Sequence analyses: Sequence reads of each segment of isolated virus was assembled using DNASTAR Lasergene SeqMan PRO version 16 software (trial version) with default parameters (Match size: 12, Minimum match percentage: 80, max. added gap in contig: 70, gap penalty: 0 gap length penalty: 0.7). Each gene segment was inspected for the presence of marker amino acids by comparing with the lists of marker amino acids that have been described in the literature so far.

Phylogenetic trees were constructed in the MEGAX program using the Neighbor Joining method in which the evolutionary distances were determined by the Maximum Composite

Likelihood method including both transitions and transversion. Uniform rates among sites were assumed and patterns among lineages were set as homogeneous. Gaps in the alignments were treated with pairwise deletion and bootstrap test was performed with 1000 replicates. Resulting trees were modified in FigTree (version 1.4.4) in order to collapse the clades which are from the same geographical origin.

3.8 Limitations of the Study

Environmental samples contain many organic and inorganic compounds that may interfere with the downstream procedures. Viruses may attach to these molecules; thus, nucleic acid extraction and/or virus isolation might be affected negatively.

3.9 Ethics Committee Approval

In this study, samples were collected from fresh droppings of wild aquatic birds without any contact with the birds. Collected samples were injected into ECEs after molecular analysis. According to legislation published in the Official Gazette (no: 28914) in 15.02.2014 laboratory animals were defined as "free living or reproducing larvae, cephalopods and vertebrates including mammals except humans after 1/3 of their embryonic development". ECEs used in this study are sacrificed before they reach 1/3 of their embryonic development. Therefore, they are not considered as laboratory animals and ethics committee approval is not required.

3.10. Legal permits

Scientific research permit for sample collection from Izmir Bird Paradise: ***No: 72784983-488.04-174660; 15.08.2017.*** The Republic of Turkey Ministry of Forestry and Water Affairs (new name Republic of Turkey Ministry of Agriculture and Forestry) on 15.08.2017.

Scientific research permit for isolation and molecular characterization of IAVs: ***No:71037622-325.01-E.3135467; 14.10.2019.*** Republic of Turkey Ministry of Agriculture and Forestry.

4. RESULTS

4.1. Prevalence of avian IAVs in Izmir Bird Paradise/Gediz Delta

In order to determine the prevalence of avian IAVs in Izmir Bird Paradise/Gediz Delta, we collected 500 fresh fecal droppings of wild aquatic birds in 6 months between December 2017 and June 2018. Among these samples 305 belonged to Charadriiformes, 79 belonged to Anseriformes. Two samples were collected from western marsh harriers immediately after defecation. Species origin of 144 samples were unknown (Table 11). Field study was not carried out in February 2018, since the weather conditions were not suitable. Each sample was collected in separate tubes containing viral transport media (PBS, glycerol, and antibiotics; Table 6), and kept in a cooler box during field studies. Upon arrival at the laboratory, all samples were frozen on dry ice to minimize the loss of viable viruses and stored in -86^{o}C freezer for further analysis.

Table 11. Number of samples collected from respective origin and distribution of RT-PCR positive samples

Origin of the fecal samples	Number of samples	Positive samples	Ratio of IAV positive samples
Unknown	114	44	38.6%
Anseriformes	79	13	16.46%
Charadriiformes	305	98	32.13%
Western Marsh Harrier	2	1	50%

Viral RNAs from fecal samples were extracted using QIAMP Viral RNA Mini Kit or RNeasy Mini Kit (QIAGEN) and the initial viral presence was investigated based on the amplification of 234bp region of M gene (30-264nt) (M-partial). The M gene was selected for the detection of IAVs since it is the most conserved gene of IAVs. Amplifications were performed using One-Step RT-PCR kit (QIAGEN), optimizing the reaction volume to 10µl. Among all 500 fecal samples, 156 were detected to be virus-positive via RT-PCR (Figure 14 and Appendix 1). Most of the positive results were obtained from the fecal samples of Charadriiformes (32.13%), to which majority of the samples belong. Fecal samples of unknown

bird origin yielded 44 positive results (38.6%) and the fecal samples of Anseriformes yielded 13 positive results (16.46%) based on the amplification of a 234 bp region of M gene via RT-PCR. Additionally, two fecal samples were sampled from western marsh harriers and one sample was positive (Table 11).

Then, M-partial positive samples were examined to determine whether they are of H5 or H7 subtypes. For that, RT-PCR reactions were set using H5 and H7 specific primers. None of the samples were detected to be of H5 or H7 subtype.

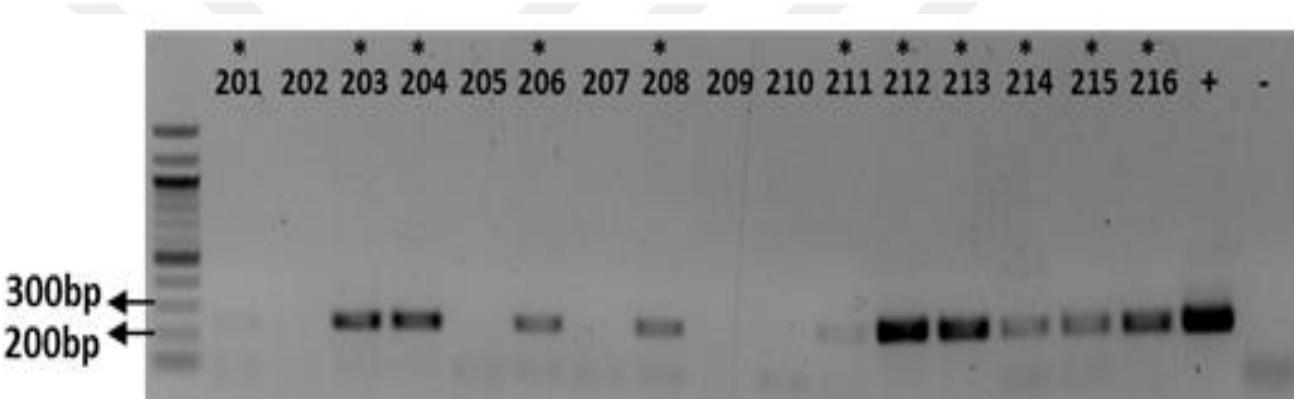


Figure 14. Representation of RT-PCR positive samples in gel electrophoresis. 234 bp region of M gene was amplified for each sample and analyzed in gel electrophoresis. Fecal samples 201-216 are shown as representatives. Virus-positive samples are indicated with "*". RNA of human H1N1 isolate, kindly provided by Prof. Dr. Candan Çiçek, Ege University, Department of Medical Microbiology, was used as the positive control. Nuclease free water was used as the negative control. 100bp DNA marker was used to identify the sizes of bands.

Our results indicate that the overall prevalence of avian IAVs between December 2017 to June 2018 in Izmir Bird Paradise/Gediz Delta was 31.2% based on the amplification of 234 bp region of M gene. The highest prevalence was observed in March 2018 (39.83%) and it was followed by January 2018 (39%), and December 2017 (37.33%). The prevalence of avian IAVs were relatively lower in April (15.15%), May (29.73%) and June (14.71%) (Figure 15).

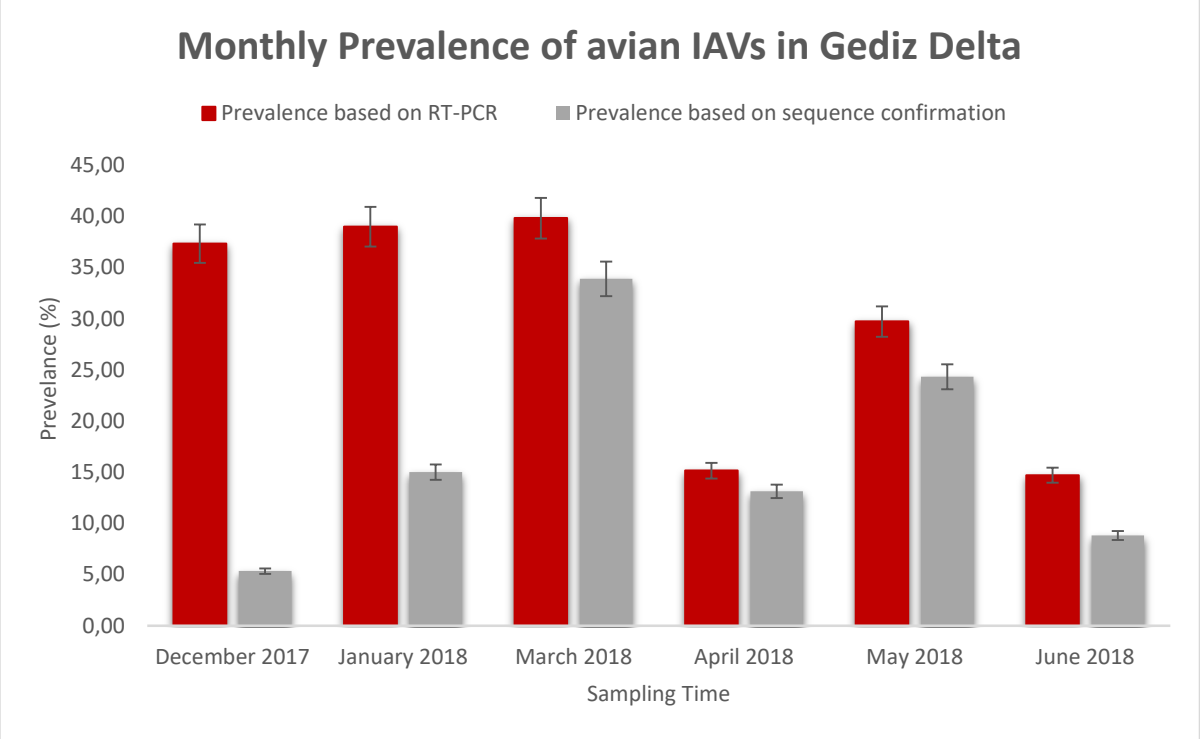


Figure 15. Monthly prevalence of avian IAVs in Izmir Bird Paradise/Gediz Delta. Overall prevalence of IAVs in the Izmir Bird Paradise/Gediz Delta region was 31.2%. The highest prevalence was observed in March 2018 (39.83%) and the lowest prevalence was observed in June 2018 (14.71%) based on RT-PCR results (red bars). The overall prevalence based on sequence confirmation was 18.6%, peaking in March 2018 (33.9%). Error bars indicate 95% CI.

Since the working materials were environmental samples, we wanted to confirm our RT-PCR results via Sanger sequencing. For that, 30µl RT-PCR reactions of 156 samples were set. Among 156 samples, 93 sample yielded enough amplicons to be purified and to be sent for sequencing. Sequence reads were confirmed to be of M gene of IAVs using NCBI BLASTn program. The analysis showed that 89 of the M-partial sequences were highly similar (>95%) to M gene sequences of IAVs found in NCBI database (Table 12, Appendix 2). Similarities of four samples (F126, F293, F295, F308) were in between 90%-94% and only one sample (F221) had ~85% similarity with the M gene of an IAV. Based on sequence results, the overall prevalence of IAVs in Izmir Bird Paradise/Gediz Delta was 18.6%. The highest prevalence was detected in

March 2018 (33.9%). Although IAV prevalence followed a similar pattern in both RT-PCR and sequence-based prevalence, the lowest prevalence was observed in December 2017 based on sequence confirmation (Figure 15).

Table 12. First BLAST (NCBI) hits for the M-partial sequences (234 bp) of representative virus-positive samples

	First BLAST Hits	Accession numbers	Percent identity
F201	Influenza A virus (A/mallard/Maine/UGAI16-5445/2016(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT421614.1	97.80%
F203	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.17%
F204	Influenza A virus (A/environment/Maryland/12OS1478/2012(H3N6)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY191124.1	97.21%
F206	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	97.56%
F208	Influenza A virus (A/mallard/Maine/UGAI16-5482/2016(H4N6)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT421638.1	97.09%
F212	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.20%
F213	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.66%
F214	Influenza A virus (A/mallard/Ohio/14OS2751/2014(H10N3)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KY463815.1	97.17%
F215	Influenza A virus (A/ruddy turnstone/Delaware Bay/437/2014(H12N4)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK828141.1	98.04%
F216	Influenza A virus (A/northern pintail/Ohio/14OS2210/2014(H5N9)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KY463895.1	98.52%

* “F” in the sample names stands for fecal and the numbers stand for the sample numbers in terms of collection order.

4.2 Isolation of avian IAVs

After identification of IAV-positive samples via molecular methods, 156 samples were inoculated into 10-days old ECEs in triplicates for virus isolation. In each round of inoculation, 3 eggs were inoculated with PBS as negative control. After 72 hours of incubation, allantoic fluids were collected and virus presence in each ECE was tested via hemagglutination assay (Figure 16). Positive results were observed in four samples from which 10 to 15 aliquots were stored at -86^{o}C. HA negative samples were re-inoculated in ECEs up to 3 passages to achieve virus isolation. However, no other IAVs could be isolated.

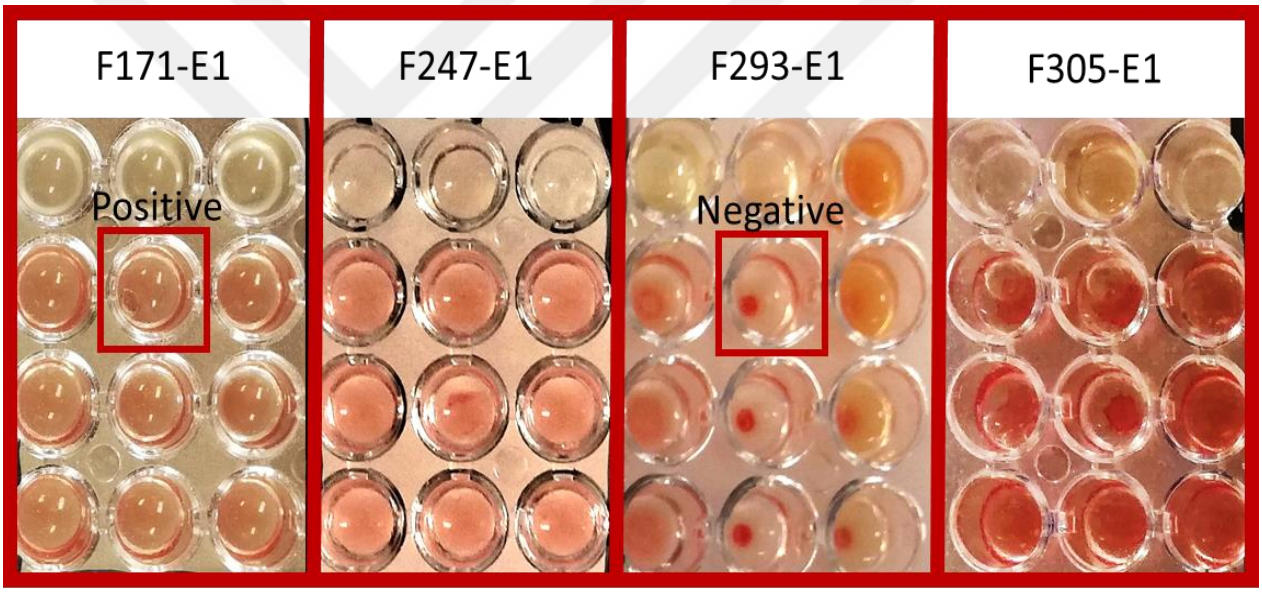


Figure 16. Confirmation of virus propagation via hemagglutination assay. Appearance of positive and negative samples are indicated with red squares. In positive samples viruses attach red blood cells and prevent them from precipitating, resulting in a homogeneous appearance. In negative samples, red blood cells precipitate at the bottom of the U-bottom plate and form a red dot. E1 demonstrates the number of egg passages (Passage 1) for the virus propagation. The viruses were propagated from the samples F171, F247, F293 and F305 during the first passage in ECEs.

4.3. Determination of subtype and titer of the isolated virus

Viral RNA was extracted from the allantoic fluid of the ECEs that were inoculated with the sample F171 and 50µl RT-PCR reactions were set up for the HA2 region of HA gene (HA1144-HA-1770R; ~680bp) and full length NA (NA-1F-NA-1413R; ~1415bp) gene (Table 4). HA2 region is highly conserved among 16 HA subtypes, thus it was used to determine HA subtype (Phipps *et al.*, 2004). After amplification, each amplicon was purified and sent for sequencing. NCBI BLAST analysis of each sequence showed that the subtype of the virus was H6N2 (Table 13). The virus isolate obtained from the sample F171 was named as "A/Aquatic bird/Gediz Delta/1/2018 (H6N2)" and abbreviated as GD/18 from here on.

Table 13. First BLAST (NCBI) hits of HA-partial and NA sequences of the virus isolate

Sample	BLAST hit for HA-partial gene	BLAST hit for NA gene
F171-E1	Influenza A virus (A/mallard duck/Georgia/7/2015(**H6**N1)) segment 4 hemagglutinin (HA) gene, complete cds	Influenza A virus (A/mallard duck/Netherlands/18/2012(H4**N2**)) segment 6 neuraminidase (NA) gene, complete cds

After isolation, HA titer and EID_{50} of GD/18 was measured. HA titer was calculated based on the highest dilution at which hemagglutination was observed. The HA titer of GD/18 was found to be 64. EID_{50} was calculated based on the virus dilution at which 50% of the inoculated ECEs were infected. The infectivity titer of GD/18 was calculated as $10^{6.5}$ EID_{50}/ml.

4.4 Genome analysis of the isolated virus

4.4.1. Molecular characterization

Each gene segment in the genome of the isolate GD/18 was amplified with gene specific universal primers (Hoffmann *et al.*, 2001) (Table 4) and each amplicon was purified by gel extraction. Purified amplicons were sent for Sanger sequencing with primers specific to each segment (Table 5). Then, the sequence reads were assembled to obtain contigs for each gene segment using DNASTAR Lasergene SeqMan PRO version 16 software (trial version). The 5' end of each assembled contig was trimmed and translated into amino acid sequences to investigate the amino acids at critical positions in each gene segment in terms of host preference,

pathogenicity, transmissibility, increased replication ability and better immune evasion. The GenBank accession numbers for the gene sequences of GD/18 are MT783429-to-MT783436 in the order of PB2, PB1, PA, HA, NP, NA, M and NS.

In order to detect experimentally defined or naturally occurring marker amino acids in each gene, an extensive literature search was conducted. Marker amino acids identified in the genome of GD/18 are listed in Table 14.

Seven marker amino acids were detected at the positions 63, 89, 309, 339, 477, 495 and 676 of PB2 gene. Six of these markers (V89, D309, K339, G477, V495, T676) were shown to increase replication ability and virulence in mice when they coexist (Li *et al.*, 2009). The other marker, I63, is shown to cause pathogenicity in mice when it is present with T677 in PB1 (Li *et al.*, 2011).

PB1 gene harbored seven amino acid markers V3 (with N328 and N375), P13, Y436, V473 and T677. All of these markers were related with increased polymerase activity and virulence in mammals based on the experiments in mouse model (Govorkova *et al*., 2005; Salomon *et al*., 2006; Gabriel *et al*., 2007; Hulse-Post *et al*., 2007; Xu *et al*., 2012), however one of the markers (Y436) was also correlated with increased polymerase activity in mallards (Hulse-Post *et al*., 2007). Additionally, amino acids at two positions, P13 and N375, were shown to be mammalian host markers (Gabriel *et al.*, 2007). The other product of segment 2, PB1-F2, contains S66 and L82 which are correlated with pathogenicity in mammals and humans, respectively (Conello *et al.*, 2007; Alymova *et al.*, 2007).

Another polymerase complex protein, PA, had four markers. One of them, A37, was shown to increase pathogenicity in mammalian cells (Yamayoshi *et al.*, 2014). The other three markers S277, Q278, P653 were correlated with adaptation to mammalian hosts (Mei *et al.*, 2016). PA-X which is also expressed from segment 3 had two markers, P28 and S65, which result in increased shut down of host translation machinery in mammals which is required for efficient expression of viral proteins (Oishi *et al.*, 2019).

Table 14. List of previously described marker amino acids that are also detected in the genome of GD/18

Gene	Marker amino acid	Phenotype	Reference
PB2	I63 (with PB1 T677)	Pathogenic in mice	Li *et al.*, 2011
	V89 (with, D309, K339, G477, V495, T676)	Enhanced polymerase activity, Increased virulence in mice	Li *et al.*, 2009
PB1	V3 (with N328 and N375)	Increased polymerase activity, Increased virulence in ferrets and mice	Govorkova *et al.*, 2005; Salomon *et al.*, 2006
	P13	Increased polymerase activity, Adaptation to mice	Gabriel *et al.*, 2005
	Y436	Increased polymerase activity and virulence in mallards, ferrets, and mice	Hulse-Post *et al.*, 2007
	V473	Increased polymerase activity and replication efficiency in mammalian cells	Xu *et al.*, 2012
	T677 (with PB2 I63)	Pathogenic in mice	Li *et al.*, 2011
PB1-F2	S66	Increased virulence in mice	Conenello *et al.*, 2007 Conello *et al., 2010*
	L82	Increased pathogenicity in mice	Alymova *et al.*, 2011
PA	A37	Significantly increased viral growth and polymerase activity in mammalian cells	Yamayoshi *et al.*, 2014
	S277	Adaptation to mammalian hosts	Mei *et al.*, 2016
	Q278	Adaptation to mammalian hosts	Mei *et al.*, 2016
	P653	Adaptation to mammalian hosts	Mei *et al.*, 2016
PA-X	P28	Increased host shutdown in	Oishi *et al.*,

		mammalian cells	2019
	S65	Increased host shutdown in mammalian cells	Oishi *et al.*, 2019
HA[a]	A156 (173) [b]	Increased virus binding to α2–6, transmissibility in Guinea pigs	Gao *et al.*, 2009; Gu *et al.*, 2017
NP	V105	Adaptation of duck origin IAVs to chickens	Tada *et a*l., 2011
NA	T117	Reduced susceptibility to oseltamivir and zanamivir	Kode *et al*., 2019
M1	D30	Increased virulence in mice	Fan *et al*., 2009
	A215	Increased virulence in mice	Fan *et al*., 2009
NS1	S42	Increased virulence in mice, Antagonism of IFN induction	Jiao *et al*., 2008
	F138 (with ESEV in PDZ binding domain)	Increased replication in mammalian cells, decreased interferon response	Fan *et al*., 2013
	A149	Increased virulence and decreased interferon response in chickens	Li *et al*., 2006

[a]Amino acid positions of HA were designated in H5 numbering.
[b]Positions in the parenthesis indicates respective position in H6 of GD/18

One of the major antigens of IAVs, HA, harbors one marker, A156 which is associated with increased virulence in mammals (Gao *et al.*, 2009). A156 also correlates with increased binding to mammalian type SA receptors and shown to be related to contact transmission between guinea pigs (Gu *et al.*, 2017).

NP gene of GD/18 contains one marker amino acid, valine at positions 105 which was shown to cause adaptation of duck origin IAVs to chickens (Tada *et al.*, 2011).

The other antigenic gene of IAV, NA, harbors one marker, T117, which causes reduction in efficiency of NA inhibitor-based antivirals, oseltamivir and zanamivir (Kode *et al.*, 2019). Apart

from T117, NA gene showed variance at some other amino acid positions. However, no critical functions were appointed to these variations in the literature.

M1 open reading frame of segment 7 had two markers (D30, A215) correlated with increased virulence in mammals (Fan *et al.*, 2009). No marker was detected in M2 which is the other protein expressed from segment 7.

NS1 contains three marker amino acids. These markers provide increased replication in mammals (S42) or chickens (A149), and increased ability to interfere with the host immune response via antagonizing IFN induction (Jiao *et al.*, 2008; Li *et al*., 2006). NS1 also contains a PDZ binding motif that accommodates binding to pro-apoptotic protein Scribble and inhibits its function thus, prevent apoptosis and ensure the progression of viral life cycle (Liu *et al*., 2010). The PDZ motif of GD/18 was "ESEV" which is common for avian viruses (Obenauer *et al.*, 2006) and highly pathogenic H5 viruses isolated from humans (Wang *et al*., 2020). GD/18 harbors phenylalanine (F) at the position 138 which increases the replication ability of the virus together with avian type PDZ binding motif (Fan *et al.*, 2013). No markers were detected in NEP which is the other protein encoded by segment 8.

4.4.2. Phylogenetic analyses of the HA and NA genes

In order to identify the origins of antigenic genes of GD/18, H6 (n=242) and N2 (n=1786) sequences were downloaded from IRD. Redundant N2 sequences were eliminated from the data set leaving 423 N2 sequences. H6 and N2 genes were aligned using BioEdit (Hall, 1999). The alignment was uploaded on the MEGAX program to construct the phylogenetic tree using the Neighbor Joining method where evolutionary distances were calculated using Maximum Composite Likelihood method. Bootstrap test with 1000 replicates were applied on each tree and bootstrap consensus trees were edited in FigTree (Rambaut, 2018).

HA gene of the GD/18 was highly similar to the H6 viruses isolated from a mallard (H6N2) and a domestic duck in Georgia (H6N1) in 2015 (Figure 17). These viruses were genetically distinct from H6 viruses detected in South and East Asia in 2016-2017. These two clusters diverged from H6 viruses detected in China in 2015. This suggests that the geographical origin of the segment was East Asia.

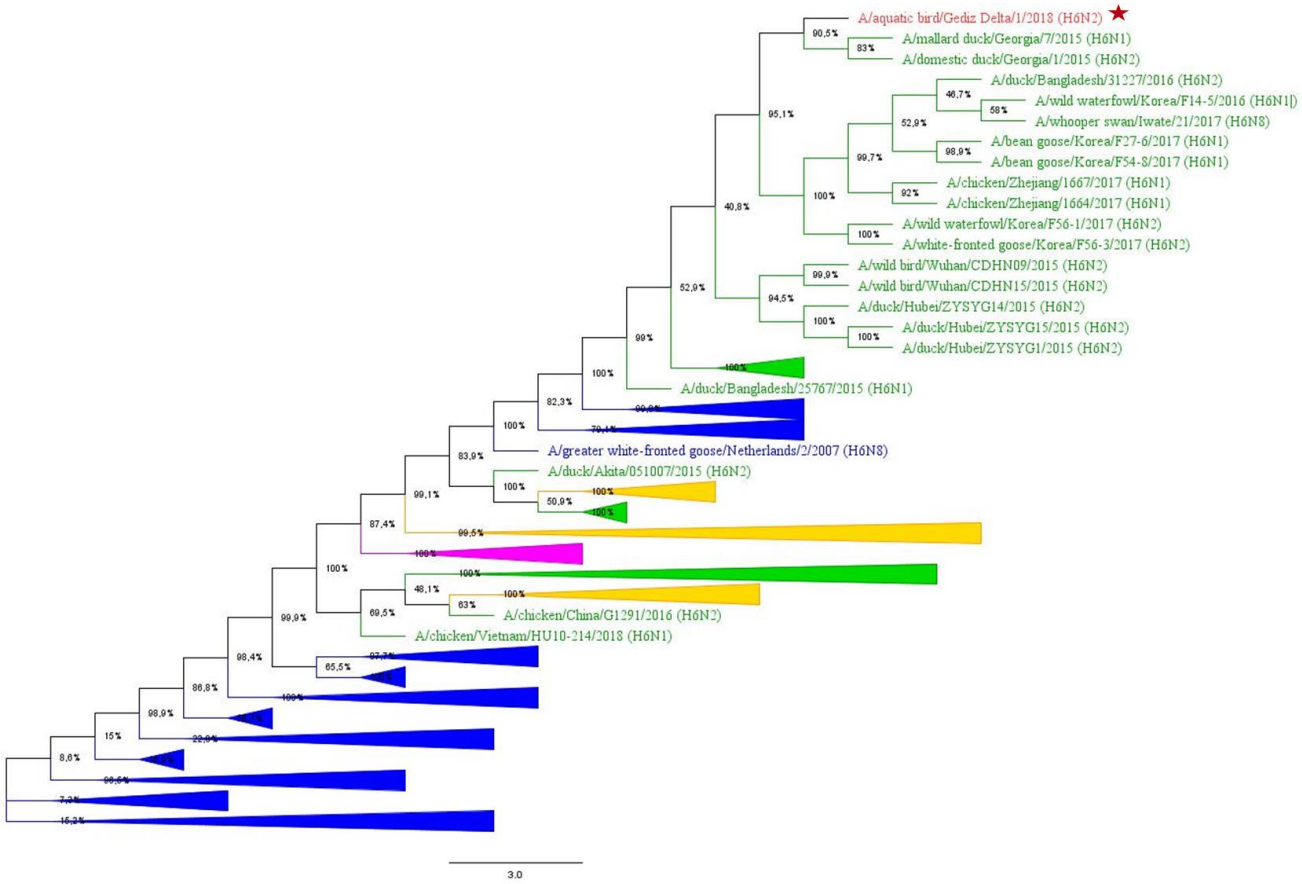


Figure 17. Origin of HA segment of GD/18. The phylogenetic tree was constructed using Neighbor Joining method. Bootstrap consensus tree is formed from 1000 replicates. Bootstrap values are given on the tree as percentage. Taxa were colored based on their geographic origins: Blue-Europe, yellow-North/South America, green-Asia, purple-Africa, red-GD/18. GD/18 was also designated with a red star.

NA gene of GD/18 was highly similar to an H6N2 virus from China detected in a goose in 2014 and clustered with N2 genes of both Asian and European viruses of Anseriformes origin (Figure 18). The cluster shares a common ancestor with N2 viruses from Europe and Africa. This divergence probably occurred before 2010 via mingling of birds from different origins at which two or more flyways intersect.

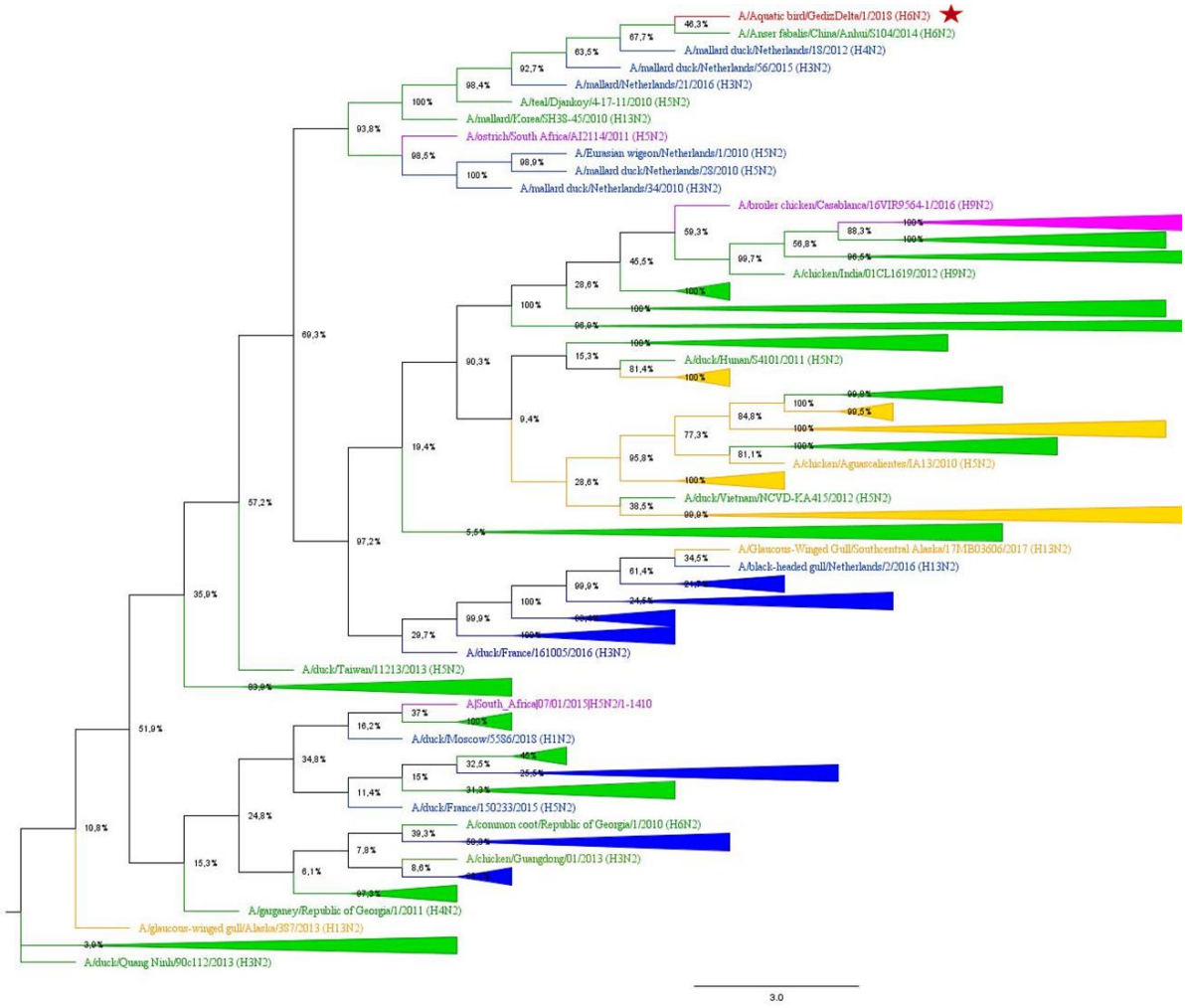


Figure 18. Origin of NA segment of GD/18. The phylogenetic tree was constructed using Neighbor Joining method. Bootstrap consensus tree is formed with 1000 replicates. Taxa were colored based on their geographic origins: Blue-Europe, yellow-North/South America, green-Asia, purple-Africa, red-GD/18. GD/18 was also designated with a red star.

5. DISCUSSION

In this thesis project, the prevalence of avian IAVs in Izmir Bird Paradise/Gediz Delta was determined using molecular methods. Four viruses from IAV positive samples were isolated and molecular markers in the genome of one virus, GD/18, were identified. Additionally, origins of the antigenic segments of GD/18 were determined via phylogenetic analyses.

Between December 2017 and June 2018, 500 fresh fecal droppings of wild aquatic birds were collected. Before sampling, areas where multiple bird species are present in high numbers were detected using binocular scopes. Samples were collected without any contact with the birds, thus, species of the birds that feces belong to was unknown. There are several methods to identify bird species from fecal material. One method involves analysis of cytochrome oxidase I (COI) gene sequence (Cheung *et al.*, 2009). In this method nucleic acid is extracted from the sample, COI gene is amplified and sequenced. However, this method is labor intensive and requires additional funds. Thus, detection of host species was not in the scope of this study. Instead, the fecal samples were attributed to Anseriformes and Charadriiformes in general based on their appearance, as the supervisor of this thesis has an extensive field experience. Sampling was performed only in Izmir Bird Paradise, a part of Gediz Delta. However, due to frequent relocation of birds for feeding and resting, the sampling site was able to represent the entire Delta.

IAV prevalence in the Delta was determined via RT-PCR and results were confirmed via Sanger sequencing. RT-PCR results indicated that 156 of 500 samples were positive based on the amplification of M-partial gene (234 bp). The amount of amplicons varied among samples. This can be a result of variance in the amount of virus found in the sample. Another reason can be the chemical and organic composition of the feces (Warren *et al.*, 1966). Among all, 93 of the samples yielded enough amplicons to be purified and sent for sequencing. Sequence analyses indicated that most of the sequences had more than 95% similarity with M genes of IAVs. Similarities of four sequences (for the samples F126, F293, F295 and F308) were between 90% and 94%. Only one sequence had ~85% (F221) similarity which might be the result of the poor sequence quality. M gene was used in this study to determine IAV prevalence via RT-PCR and sequencing since it is the most conserved gene of IAVs hence, M gene sequences are highly similar among all IAV subtypes. Therefore, first BLAST hits of some M-partial sequences were M genes of H5/H7 sequences although viruses were not of these subtypes. Additionally, some of the M-partial sequences hit mammalian M gene sequences. Since the part of the M gene that was amplified and sequence was too short and does not contain any species-specific nucleotide polymorphism, these findings does not correlate with the species origin of the detected viruses.

The monthly distribution of the prevalence of avian IAVs was between 37% to 39.83% in the first three sampling dates, however there was a decrease to 15.15% in April 2018. The

prevalence increased once again to 29.73% in May 2018, then, it decreased to the lowest value detected in the study in June 2018 (14.71%). This trend in IAV prevalence seems to be in parallel with the abundance and diversity of the aquatic birds in sampling site (Izmir Bird Paradise/Gediz Delta). Winter migrants arrive in the area at the beginning of winter and leave around late March. This coincides with the decrease of avian IAV prevalence in April 2018. In the mid/late spring summer migrants begin to arrive at the area and reach their peak numbers in May which also coincides with the increase in avian IAV prevalence in that time.

After detection of prevalence, avian IAV positive samples which were identified via molecular methods were inoculated into 10 days old ECEs in triplicates. The inoculation of RT-PCR positive samples resulted in the isolation of four viruses. The virus isolation rate was low (2.56%), compared to our detection rate via molecular methods. However, isolation of avian IAVs, from environmental samples often results in low isolation rates. In one study, the isolation rate of IAVs was 6.25% from samples which were detected to be positive via molecular methods (Araujo *et al.*, 2018). In another study, 13 viruses were isolated from 280 qPCR positive samples (4.64%) (Indriani *et al.*, 2010). The challenge in isolation of avian IAVs from environmental samples might be due to the composition of the sample material. Fecal samples were collected from sediments of shallow parts of ponds or from the shores of ponds and Aegean Sea, thus some sediment or soil was collected with the samples. The chemical and microbial composition of the sediment might interfere with the isolation of avian IAVs and other viruses from environmental samples (Warren *et al.*, 1966). It was also suggested that low yields in virus isolation compared to detection via molecular methods might be related with the viability of the virus in the sample (Indriani *et al.*, 2010).

The subtype of one isolate was identified via sequencing the HA2 region of HA, and full length of NA. Based on sequence identities in BLAST analyses, the subtype of the isolate was determined as H6N2 and named as A/Aquatic bird/Gediz Delta/1/2018 (GD/18). IAVs with H6 subtype are one of the most prevalent viruses detected in avian species (Cumming *et al.*, 2011; Huang *et al.*, 2012; Kumar M. *et al.*, 2018; Araujo *et al.*, 2018; Zanaty *et al.*, 2019, Hassan *et al.*, 2020). It was shown that avian H6 viruses were able to transmit and infect mammals without prior adaptation (Gillim-Ross *et al.*, 2008; Koçer *et al.*, 2012). H6 viruses of avian origin were also shown to be able to transmit between guinea pigs via direct contact (Qu *et al.*, 2017).

Antibodies for H6 viruses were detected in humans indicating that H6 viruses can also transmit to humans (Myers *et al.*, 2007; Xin *et al.*, 2015). This finding was validated by the isolation of an H6N1 virus from a hospitalized patient (Wei *et al.*, 2013). Genetic analyses indicated that the virus was closely related to H6 viruses circulating in poultry. These studies indicate that avian H6 viruses are capable of interspecies transmission.

After the isolation, the whole genome of GD/18 was amplified, purified, and sequenced using gene specific primers. 30 amino acid markers were identified in all genes of GD/18, except M2 and NEP (Table 14, Appendix 3).

The first barrier that avian IAVs should overcome to infect mammals is the low affinity of HA of avian IAVs to bind mammalian type SA receptors. A number of mutations can alter the affinity of HA from avian type SA receptors towards mammalian type SA receptors. Two well-known markers are the amino acid changes at Q222L and G224S. Acquisition of these mutations causes alteration in HA affinity towards α-2,6 linked SA receptors; thus, enables transmission to mammals (Herfst *et al*., 2012; Song *et al*., 2017). Although these markers were not observed in our isolate, another notable marker, A156 was detected in HA of GD/18. Alanine at position 156 results in loss of glycosylation at position 158 which allows binding to α-2,6 linked SA receptors (Gu *et al.*, 2017). Important thing about this marker is that it retains the ability to bind to avian type SA receptors (Wang *et al*., 2010). Apart from its effect on SA affinity, A156 was also shown to contribute to the transmissibility of IAVs among guinea pigs via direct contact (Gao *et al*., 2009). Another feature determined by HA protein is the pathogenic character of the virus, which depends on the amino acid motif at the cleavage site of HA protein (/GLF). The H6N2 virus that was isolated in this study contains R/GLF motif at the cleavage site as expected. Presence of a single arginine amino acid before the cut site (R/GLF) depicts low pathogenicity (low pathogenic avian influenza; LPAI).

The other surface protein NA is responsible for the release of newly formed viral particles. NA cleaves HA molecules of progeny viruses which are attached on the SA residues during budding (Palese *et al*., 1974). NA has become one of the targets for antiviral drugs after increased resistance against M2 inhibitor antivirals (amantadine). To date, different NA inhibitors were produced using different strategies (Varghese *et al*., 1992; von Itzstein *et al*., 1993; Babu *et al*., 2000). Zanamivir and Oseltamivir were the first NA inhibitor antivirals, however mutations

that confer resistance to these drugs were identified not much later. One famous example, H274Y mutation (N1 numbering), was identified in experimentally infected individuals (Ives *et al.*, 2002). In a comprehensive surveillance study, histidine at position 155 of N1 gene was shown to confer resistance to both zanamivir and oseltamivir (Monto *et al.*, 2006). None of these markers were detected in NA of GD/18. However, the virus contained another marker at position 119 (N2 numbering). The presence of threonine at this position was shown to cause reduced susceptibility of HPAI H5N1 viruses to both zanamivir (11.8 fold) and oseltamivir (18.6 fold) (Kode *et al.*, 2019).

Another obstacle that avian IAVs should overcome to infect mammals is the temperature barrier that restrains polymerase complex to function efficiently. Polymerase complex of avian IAVs functions at temperatures ~41°C (digestive tract), however optimum temperature for human IAVs is ~33°C (upper respiratory tract) (Massin *et al.*, 2001). However, IAVs, due to high mutation rates of their genomes, evolve to overcome this barrier, as well. One of the most studied examples of such mutations is PB2 E627K (Subbarao *et al.*, 1993, Hatta *et al.*, 2007). Another mutation on PB2 gene that provides adaptation to mammals is D701N (Li *et al*, 2005; de Jong *et al.*, 2006). PB2 of GD/18 did not contain the markers mentioned above, however it harbors seven other markers. PB2 I63 together with PB1 T677 was shown to cause pathogenicity in mice. The interaction interphase for PB2 and PB1 is located at C terminal (first 124 amino acid residue) and N terminal domains (residue between amino acids 506 and 659) (Perales *et al.*, 1996). These two amino acid positions were located at the PB2-PB1 interaction interphase and results in increased replication efficiency and pathogenicity in mice (Li *et al.*, 2011). The other six amino acid markers also result in increased polymerase activity and virulence in mammals when they are present together (Li *et al.*, 2009). Presence of V89, D309, K339, G477, V495 and T676 can compensate the E627 (avian signature) for mammalian adaptation (Li *et al.*, 2009). Of these locations 89, 309 and 339 were suggested to involve in the interaction of PB2 with PB1, and heat-shock protein 90 (HSP90); position 477 located in nuclear localization signal and position 676 in HSP90 interaction interphase (Shinya *et al.*, 2007). The presence of these amino acids at respective places suggests increased replication and pathogenicity in mice via improving interactions with polymerase complex and host related proteins (Li *et al.*, 2009).

PB1 protein which is the base unit of polymerase complex, interacts with PB2, PA and NP at different domains. Seven markers were identified in PB1 of GD/18 including T677 which acts with I63 in PB2. Another set of amino acids act together to increase replication and virulence are V3, N328 and N375. These amino acids were identified by Govorkova *et al.* (2005) while comparing pathogenicities of lethal and non-lethal H5 viruses. Then Salomon *et al*. (2006) illustrated that co-occurrence of these markers are responsible for increased replication ability and virulence in mammals. Among these three markers V3 was also shown to increase polymerase activity of HPAI H5N1 virus (Elgendy *et al.*, 2017). Another marker that was postulated to be related with host preference was P13. This residue is located at the PB1-PA interaction interphase and may modulate a more beneficial interaction that provides better replication in mammalian cells (Gabriel *et al*., 2005; Gabriel *et al*., 2007). In a later study Dreier *et al.* (2019) showed that this marker plays a role in contact transmission of an H7 virus between Guinea pigs, thus, this marker not only increases replication ability in mammals but also contributes to the transmission of virus via direct contact. One interesting marker, Y436, was also detected in PB1 protein of the isolated virus. This marker was shown to be correlated with increased polymerase activity and virulence in mammals while retaining its pathogenicity in avian species, as well (Hulse-Post *et al*., 2007). Another marker that was also present in swine flu pandemic virus (pH1N1) was valine at position 473. V473 was conserved in pH1N1 viruses and V473L substitution decreased replication of pH1N1 in mammals. Thus, it was concluded that V473 was correlated with increased replication of the virus in mammals.

The other component of the polymerase complex, PA, functions in coordination with PB2 protein in cap snatching that permits localization of viral RNAs in host cytosol for translation. PA protein cleaves host mRNA cap structure and forms short molecules of RNA which are used as primers, as well (Fodor *et al*., 2002, Dias *et al*., 2009). Four markers were identified in PA gene. A37 increases polymerase activity of the virus in mammalian cells (Yamayoshi *et al.*, 2014). Alanine at this position also resulted in increased pathogenicity in ferrets although their titers were lower than the virus harboring S37 (Yamayoshi *et al.*, 2014). The other three marker amino acids were S277, Q278, P653. These amino acids were found to be correlated with adaptation to mice (Mei *et al.*, 2016).

NP is a multifunction protein that attaches viral RNA to form ribonucleoprotein complex by interacting with polymerase complex proteins, and takes part in replication (Biswas *et al*., 1998). NP of GD/18 contains one marker, V105. The position 105 is located at the surface of the body domain of NP where it interacts with PB2 protein (Portela and Digard, 2002). Stability of the interaction between NP and PB2 was shown to be correlated with increased replication in previous reports (Labadie *et al*., 2007; Ng *et al*., 2012). To alter the host specificity of the virus, however, PB2 K627 was still needed. On the other hand, Tada *et al.* (2011) showed that duck origin viruses which contained valine at position 105 of NP were able to replicate efficiently in chicken cells. However, they postulated that the interaction between NP and PB2 may not be a determinant of this host switch.

Segment 7 of influenza A genome encodes two proteins, M1 and M2. M1 protein is the capsid protein that encapsulates the viral genome. It also plays a role in nuclear export of vRNPs and in the packaging process. M2 protein, on the other hand, is a transmembrane protein that acts as an ion channel and regulates the release of the viral genome to the host cytoplasm. M2 protein was identified as a good target for antivirals as it was conserved among all IAVs, however, increasing numbers of resistant strains to M2 inhibitors, led to use of other proteins as targets for novel antiviral drugs. Although GD/18 contained no marker amino acids in M2, the two markers that increase pathogenicity of avian viruses in mammalian species were detected in M1. These two markers, D30 and A215 were identified by Fan *et al.*, (2009) while determining pathogenicity difference between different lethal and non-lethal H5 viruses. Both markers resulted in increase in pathogenicity individually, however combination of these two markers further contribute to the pathogenicity of the virus (Fan *et al.*, 2009).

As all pathogens, avian IAVs also developed ways to evade the host immune system. NS1 protein encoded by segment eight antagonizes the host immune system by repression of IFN response. The repression involves inhibition of host cellular factors that induce IFN response related genes such as IFN regulatory factor (IRFs) and NFκB. NS1 of GD/18 contained three markers related with increased virulence in mammals and chickens. The first marker, S42, increased pathogenicity of an avian IAV in mice. S42 was shown to almost completely abolished production of IFN-α/β production *in vitro* (Jiao *et al*., 2008). NS1 protein also functions to prevent apoptosis via binding cellular factors such as scribble, Dlg1, MAGI-1, MAGI-2, and

MAGI-3 through its PDZ binding motif (PBM) (Liu *et al.*, 2010, Golebiewski *et al*., 2011). The consensus motif at NS1 PBM is defined as "RSKV" and "ESEV" for human IAVs and avian IAVs, respectively. Avian PBM was shown to increase virulence of avian viruses in mammals and found in human isolates of HPAI H5 viruses frequently (Obenauer *et al.*, 2006; Soubies *et al*., 2010; Wang *et al*., 2020). Golebiewski *et al*. (2011) also showed that other binding motifs RSKV, KSEV, or EPEV were unable to bind the host factors mentioned above. NS1 of GD/18 virus possesses ESEV and contains another marker, F138, associated with increased virulence accompanying with the presence of ESEV (Fan *et al.*, 2013). In the study conducted by Fan *et al*. (2009) HPAI virus containing NS1 F138 was able to cause systemic infection in mice, whereas infection caused by the wild type virus (NS1 Y138) was confined in the lungs. This may illustrate that highly pathogenic character of influenza viruses may not be related solely with multi-basic amino acid insertion to HA cleavage site. The last marker contained by NS1 protein of GD/18 was A149 which was associated with increased virulence and the ability to antagonize IFN response in chickens and geese (Li *et al*., 2006). In the related study, Li *et al.* (2006) infected chickens and geese with H5N1 viruses containing NS1 A149 and observed that the virus containing the marker was able to replicate in multiple organs and kill the host while the virus without the marker did not yield any apparent disease outcome in poultry.

GD/18 also encodes several auxiliary proteins. PB1-F2 protein is a product of segment 2 and identified in 2001 by Chen *et al.* and known to contribute to induction of apoptosis. It was also postulated that PB1-F2 protein is a viral factor that provides immune evasion as it is more apoptotic in immune cells (Chen *et al*., 2001; Zamarin *et al*., 2005). A well-known marker on this protein S66 was shown to contribute to the increased virulence of 1918 pandemic virus (McAuley *et al.*, 2007). This marker which is also present in PB1-F2 of GD/18, results in delay in early innate response to enhance the virus propagation. Additionally, this marker was shown to be associated with upregulation of cytokines and chemokines related to infiltration of monocytes and neutrophils into tissue at the late phases of the infection. Thus, S66 also results in increased tissue damage (Conenello *et al*.,2007; Conenello *et al*., 2011). The other marker present in PB1-F2 of GD/18 is L82 which was shown to increase expression of proinflammatory cytokines and lead to severe lung injury in mice (Alymova *et al*., 2011). Alymova *et al*. also showed that presence of this marker promotes secondary infections by *Streptococcus pneumonia*.

The other auxiliary protein PA-X was identified by Jagger *et al*. in 2012. This protein is a +1 ribosomal frameshifting product of segment 3 and contains the endonuclease domain of PA protein. The protein is not essential for the efficient replication, but it increases the viral protein expression via selectively degrading host mRNAs. This activity of PA-X protein, host shutoff, also results in inhibition of host immune response. One way of PA-X function as an immune suppressor is to decrease expression of MHC proteins to prevent antigen presentation (Jagger *et al.*, 2012). In this study, two markers were identified in PA-X of GD/18 virus. Both P28 and S65 result in increased host shut off, while combination of these two markers further increase host mRNA decay (Oishi *et al*., 2019). Since PA-X shares the N-terminal region with PA protein, Oishi *et al*. also checked polymerase activity in the presence of these two markers. Their results indicate that polymerase activity increases in the presence of both markers with an additive effect.

Apart from genome-wide investigation of molecular markers, genetic analyses in this study involved determination of geographical origin of the antigenic genes of GD/18 via phylogenetic analyses. In order to assess the origins of HA and NA, H6 and N2 sequences from Asia, Europe, Africa and America were downloaded from IRD. The BLAST hits of H6 of GD/18 contained limited number of H6 sequences from Asia and America before 2015. Thus, sequences from these locations used in this study were limited to years 2015-2020. For the same reason N2 sequences from Asia and America was limited to years 2010-2020. In spite of the year restriction, the data set was too large. Thus, redundant sequences from Asian and American N2 sequences were eliminated using Jalview program. Then, neighbor joining trees with 1000 bootstraps were constructed where evolutionary distances were calculated with maximum composite likelihood method.

Phylogenetic analyses of HA of GD/18 indicated that the virus was originated from viruses which were circulating in China in 2015. After 2015, two clades of H6 viruses diverged. One of the clades continued to circulate in South Asia. The other clade which contains GD/18, settled in Caucasus after 2015. The introduction of the virus to Turkey might have occurred around the same time with Caucasus. However, sparsity of the surveillance data from Turkey prevents such assumptions.

NA of GD/18 clustered with N2 viruses from Asia and Europe. This clade shared a common ancestor with N2 sequences from Europe and Africa. These two clades were genetically distinct from each other and diverged from each other around 2010. This divergence was probably due to the mingling of migratory birds of Asia, Europe and Africa at the intersection points of Black Sea/Mediterranean and West Asian/East African Flyways. Since Turkey contains branches of both flyways emergence of such viruses is presumable.

The findings of this thesis study illustrate that IAVs circulating in Izmir Bird Paradise/Gediz Delta carries amino acid markers which may provide the ability to transmit to mammals including humans. Furthermore, amino acid markers which confer adaptation to poultry in NP and NS1 of GD/18 indicate that IAVs in the Delta pose a risk for poultry and increase the risk of human exposure to the virus. Thus, the virus poses a threat to both public and veterinary health.

6. CONCLUSION AND FUTURE DIRECTIONS

Avian IAVs are naturally carried by aquatic birds and they stay quiescent in their natural hosts without any apparent symptoms. However, due to their rapidly changing genome they can acquire new mutations that may provide selective advantages to the virus to cross species barriers and adapt to new hosts. Avian IAVs have contributed to the emergence of at least four pandemics and caused sporadic outbreaks that affect both public and veterinary health. Most importantly, all pandemic viruses were of low pathogenic IAVs. Thus, global surveillance studies must be conducted to monitor the virus evolution and to prevent future outbreaks.

Turkey is important in terms of IAV evolution since branches of two major flyways, Mediterranean/Black Sea and West Asian/East African Flyways, cross over Turkey. Therefore, thousands of migratory birds from different locations visit Turkey for wintering and breeding or use Turkey as a stopover location. Through migratory birds, different IAVs of different origins are introduced into the wetlands of Turkey every year. Gediz Delta, being one of the wetlands, hosts 60000-90000 aquatic birds every year. The Delta also provides shelter for many mammalian species and visited by many people for different purposes. This coexistence of various species in the Delta provides a great opportunity to IAVs to adapt to mammals and transmit humans.

Therefore, the aim of this thesis study was to detect the prevalence of IAVs, and to identify molecular markers harbored in their genomes in terms of host preference, pathogenicity, transmissibility, increased replication ability and better immune evasion. For this purpose, 500 samples from fecal droppings of aquatic birds were collected in Izmir Bird Paradise/Gediz Delta between December 2017 and June 2018. Using molecular methods, prevalence of avian IAVs in the Delta based on the amplification of partial M gene was 31.2% ranging between 14.71% to 39.83% depending on the sampling time. The overall prevalence based on the confirmation of RT-PCR results via Sanger sequencing was 18.6%. The prevalence ranged between 5.33% (December 2017) and 33.90% (March 2018).

Inoculation of positive samples into ECEs resulted in isolation of four viruses (2.56%) and the subtype of one virus was identified as H6N2 (GD/18). The whole genome of the virus was sequenced and analyzed to identify molecular markers that may provide mammalian adaptation. A total of 30 marker amino acids were identified in ten viral proteins. Most of these markers were related to increased replication and pathogenicity in mammals which means that the virus may have a potential to transmit to humans. Additionally, there were several markers for adaptation to poultry. Thus, GD/18 also poses a threat for poultry, which also increases the risk of human exposure to the virus. Although molecular markers harbored in GD/18 were identified, further *in vitro* and *in vivo* studies are required to analyze the effect of these markers.

Phylogenetic analyses revealed that the HA and NA genes of GD/18 was Anseriformes origin. HA segment was originated from Asian H6 viruses that circulated in 2015-2017. The NA gene was genetically close to Asian and European viruses that were detected in 2010-2014. This suggests that the virus might have been circulating in Turkey for a while; however, lack of surveillance data prevents us to make more precise assumption on the introduction of this virus to Turkey.

This thesis study documents the prevalence of IAVs in the Gediz Delta between December 2017-June 2018 and the isolation of an H6N2 virus from Turkey for the first time. Additionally, this is the first report that involves identification of molecular markers in the whole genome of an avian IAV from Turkey in terms of mammalian and poultry adaptation, increased pathogenicity, better replication and immune repression ability in mammals.

Extensive surveillance studies that cover entire Turkey must be conducted in order to have a more comprehensive understanding on IAV evolution in Turkey. This would also facilitate the identification of currently circulating IAV strains in Turkey that could pose threat to public and veterinary health. The data obtained from such studies will also contribute to the understanding of the general scheme of IAV evolution and will help to take necessary precautions to prevent future outbreaks.

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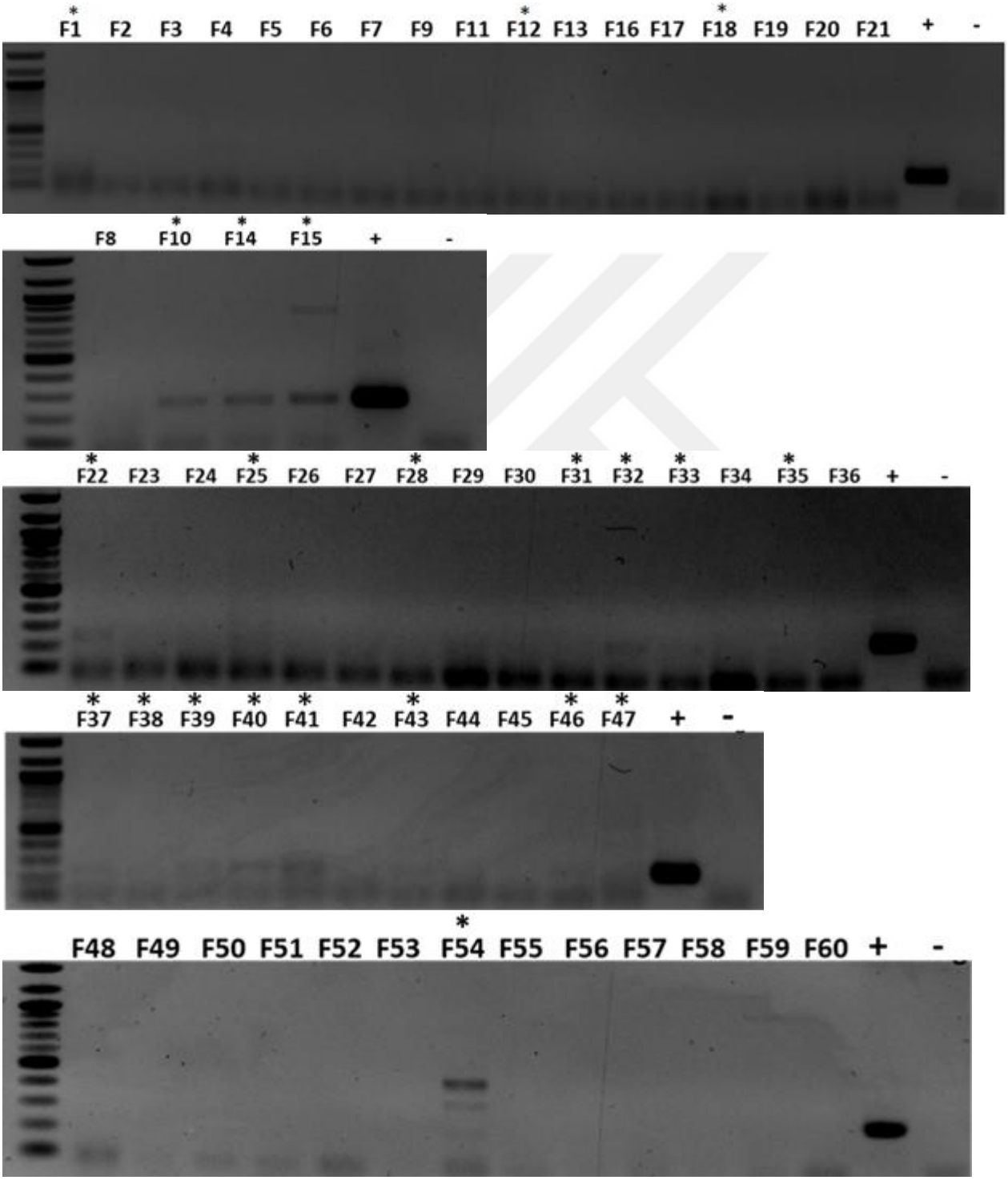
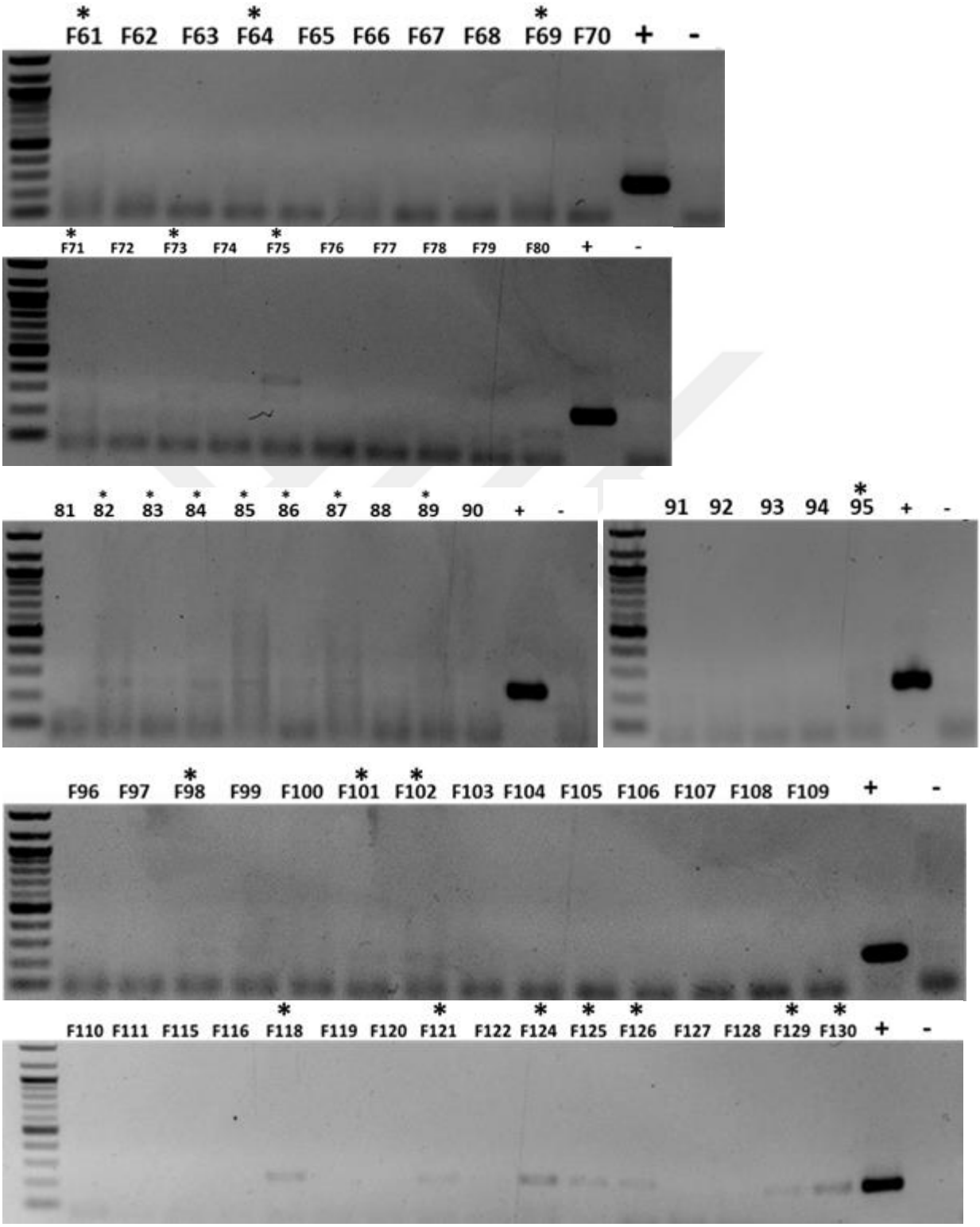
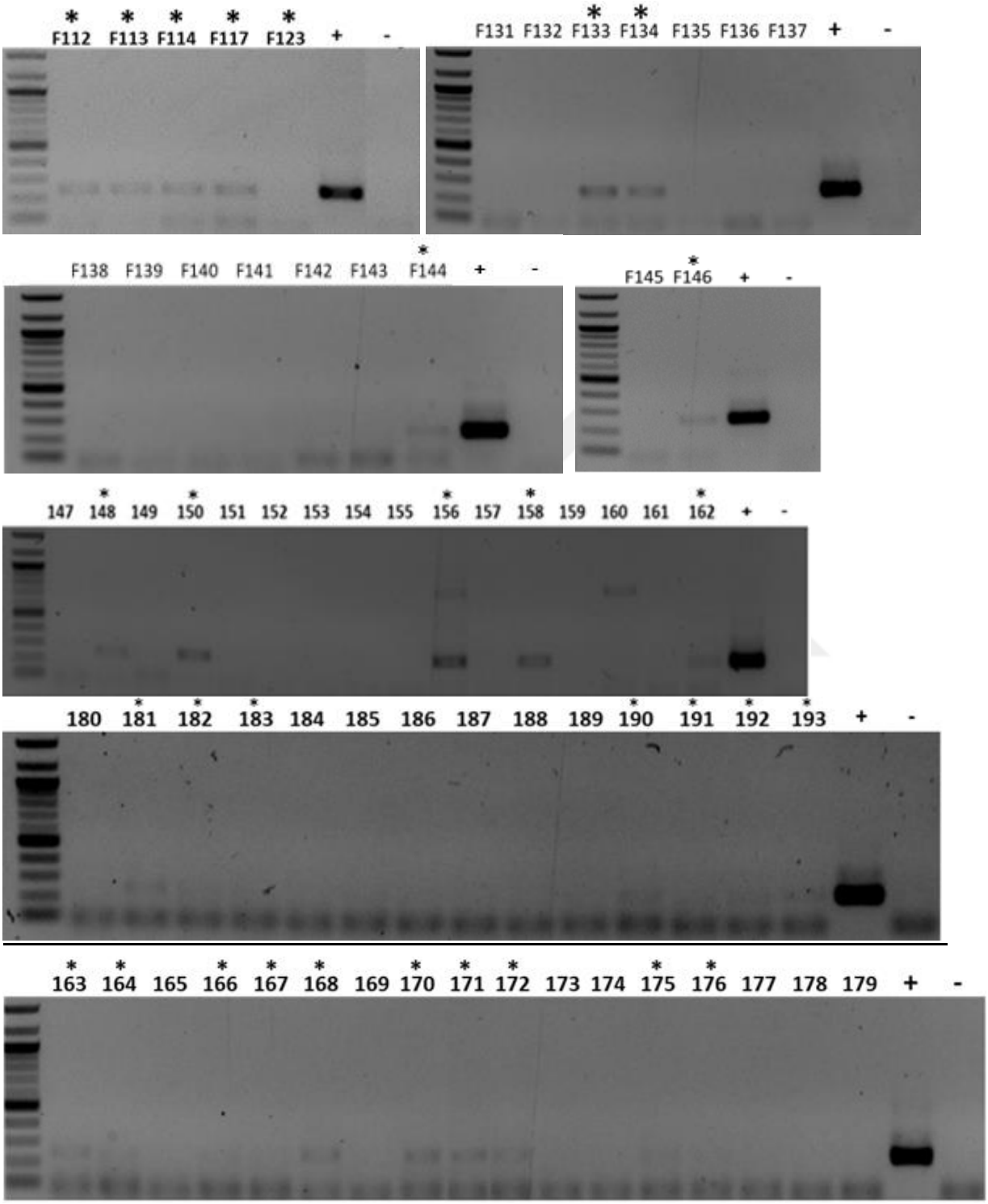
APPENDIX-1: Gel electrophoresis analyses of RT-PCR reactions representing positive samples




F61 F62 F63 F64 F65 F66 F67 F68 F69 F70 + -
F71 F72 F73 F74 F75 F76 F77 F78 F79 F80 + -
81 82 83 84 85 86 87 88 89 90 + -
91 92 93 94 95 + -
F96 F97 F98 F99 F100 F101 F102 F103 F104 F105 F106 F107 F108 F109 + -
F110 F111 F115 F116 F118 F119 F120 F121 F122 F124 F125 F126 F127 F128 F129 F130 + -


* * * * *
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* *
F131 F132 F133 F134 F135 F136 F137 + -
*
F138 F139 F140 F141 F142 F143 F144 + -
*
F145 F146 + -
* * * * * *
147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 + -
* * * * * * * * * * * *
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* *
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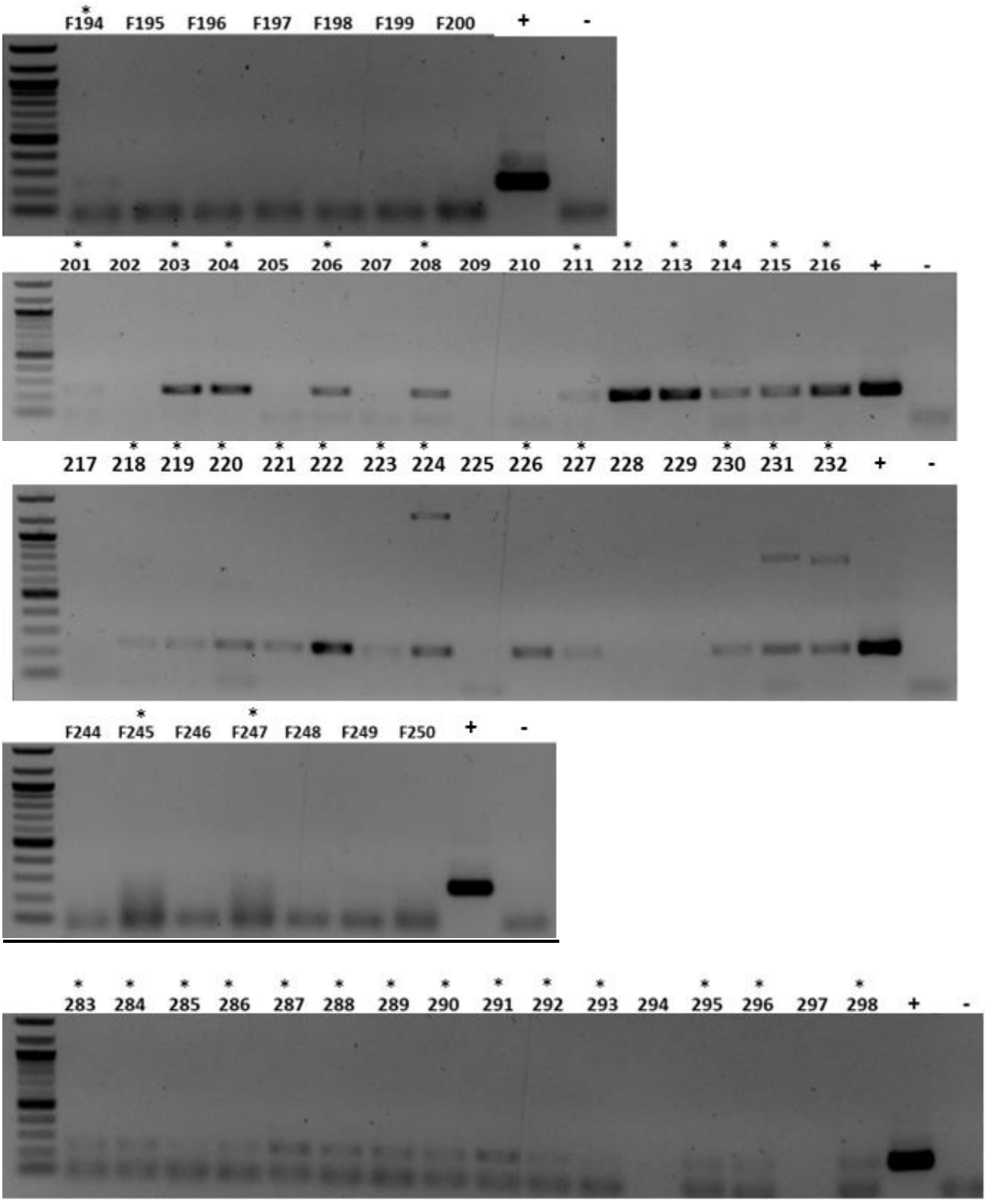
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201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 + -
217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 + -
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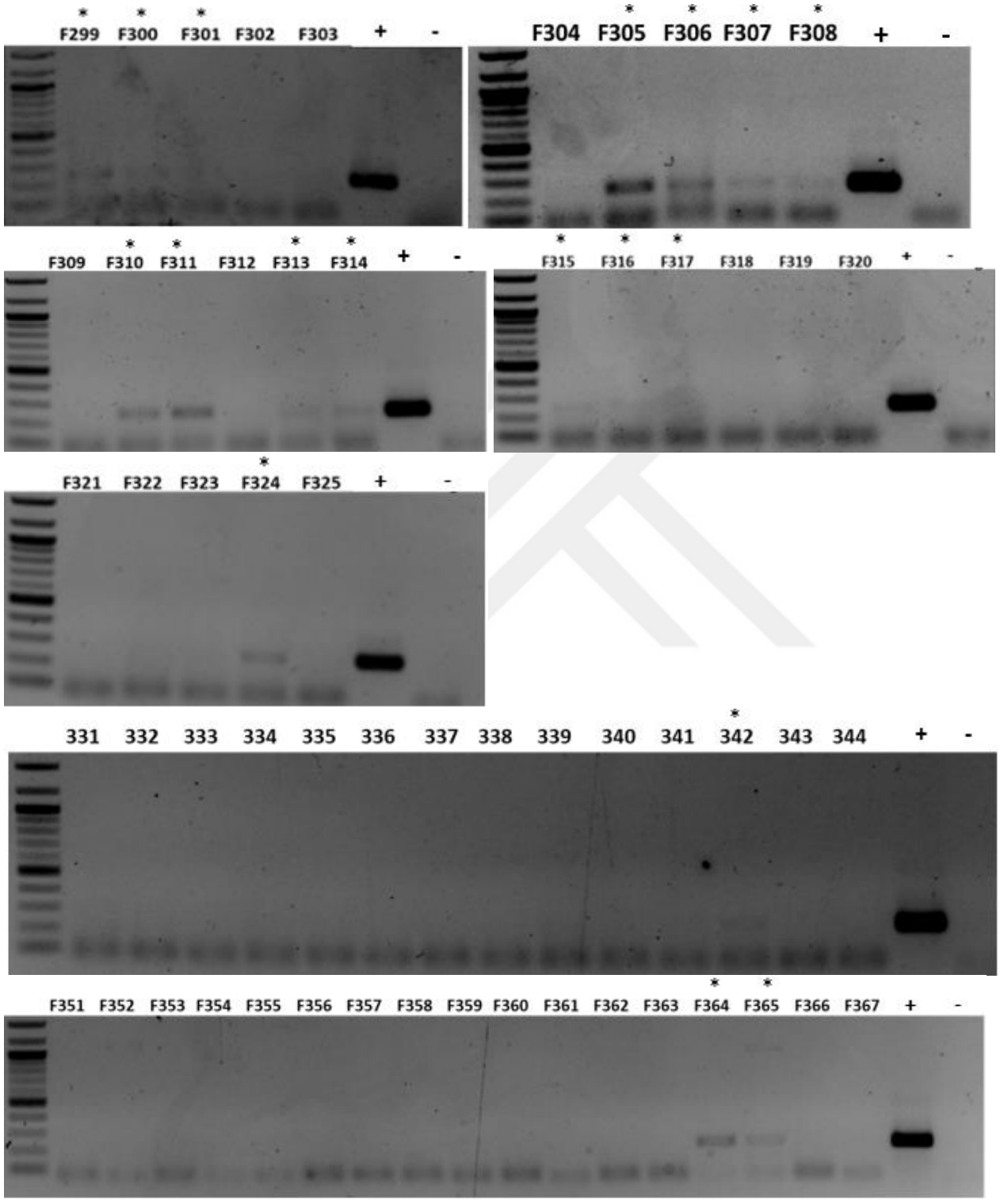
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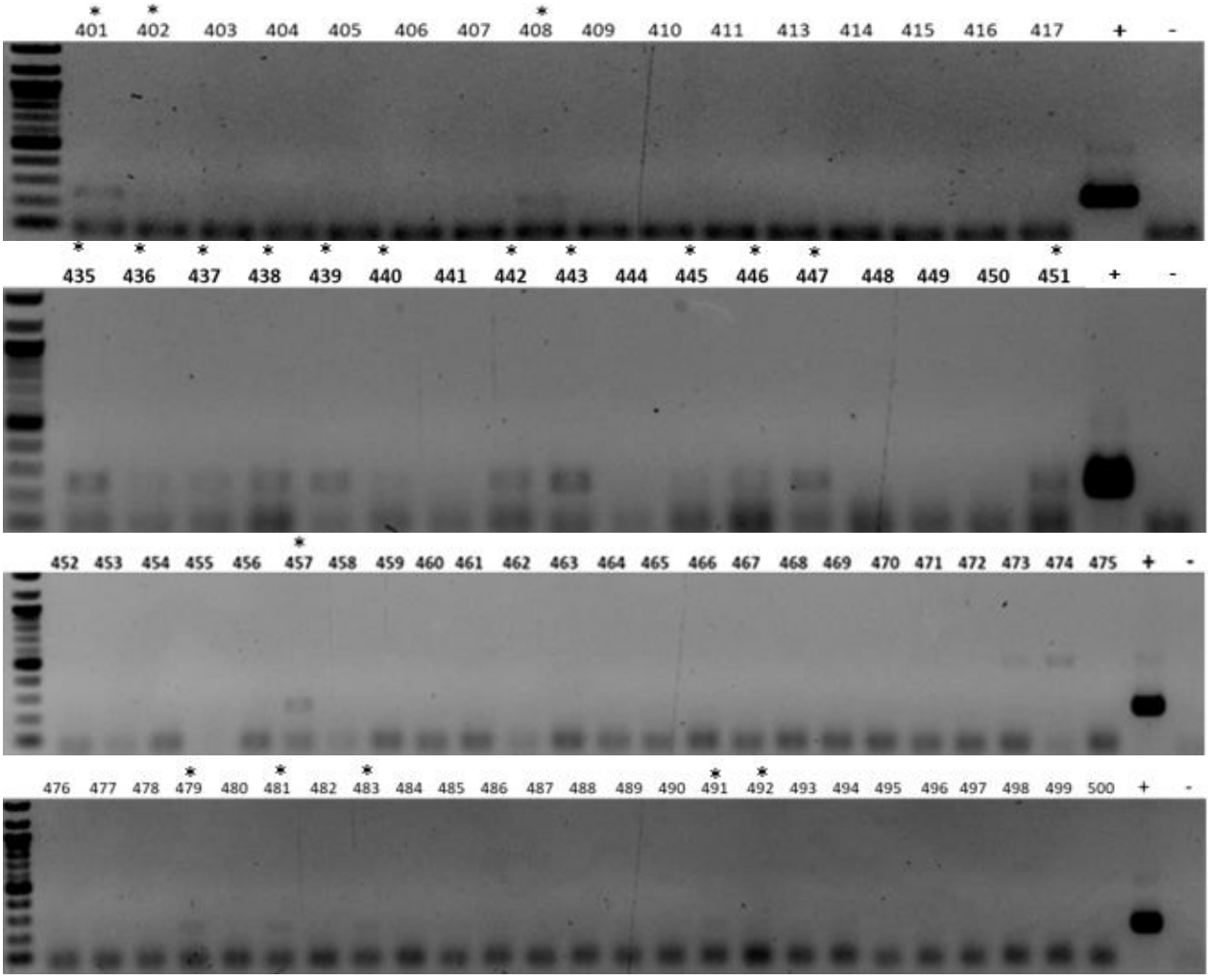

401 402 403 404 405 406 407 408 409 410 411 413 414 415 416 417 + -
435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 + -
452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 + -
476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 + -

APPENDIX 2: First BLAST hits for M-partial amplicons (234 bp region of M gene)

Sample	First BLAST Hits	GenBank Accession numbers	Percent identity
F12	Influenza A virus (A/swine/Ontario/13-1/2012(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KM489599.1	99.18%
F14	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.95%
F15	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	97.54%
F25	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	99.46%
F83	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.89%
F87	Influenza A virus (A/duck/Mongolia/667/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1)	MT020233.1	100.00%

	genes, complete cds		
F113	Influenza A virus (A/swine/Nebraska/A01774481/2016(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KX351170.1	98.75%
F114	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.43%
F118	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	97.91%
F124	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.42%
F125	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	96.81%
F126	Influenza A virus (A/swine/Manitoba/D0333/2014(H1N2)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY195576.1	90.84%

F129	Influenza A virus (A/shoveller duck/Shanghai/PD1018-32/2017(H6N5)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, partial cds	MH352246.1	95.00%
F133	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.41%
F144	Influenza A virus (A/swine/Nebraska/A01774481/2016(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KX351170.1	96.63%
F146	Influenza A virus (A/duck/Mongolia/667/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT020233.1	99.33%
F150	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.33%
F156	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	98.56%
F162	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	96.86%

F164	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.91%
F166	Influenza A virus (A/Delhi/053/2011(H1N1)) segment 7 matrix protein 2 (M2) gene, partial cds; and matrix protein 1 (M1) gene, complete cds	KP317439.1	98.80%
F167	Influenza A virus (A/Alaska/07/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK676352.1	97.41%
F170	Influenza A virus (A/Alaska/07/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK676352.1	97.41%
F171	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.11%
F172	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	97.96%
F175	Influenza A virus (A/Alaska/07/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds		97.88%

F176	Influenza A virus (A/Alaska/07/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK676352.1	97.38%
F181	Influenza A virus (A/Alaska/07/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK676352.1	97.35%
F201	Influenza A virus (A/mallard/Maine/UGAI16-5445/2016(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT421614.1	97.80%
F203	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.17%
F204	Influenza A virus (A/environment/Maryland/12OS1478/2012(H3N6)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY191124.1	97.21%
F206	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	97.56%
F208	Influenza A virus (A/mallard/Maine/UGAI16-5482/2016(H4N6)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT421638.1	97.09%

F212	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.20%
F213	Influenza A virus (A/mallard-black duck hybrid/New Brunswick/00904/2010(H10N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY139378.1	97.66%
F214	Influenza A virus (A/mallard/Ohio/14OS2751/2014(H10N3)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KY463815.1	97.17%
F215	Influenza A virus (A/ruddy turnstone/Delaware Bay/437/2014(H12N4)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK828141.1	98.04%
F216	Influenza A virus (A/northern pintail/Ohio/14OS2210/2014(H5N9)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KY463895.1	98.52%
F218	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	98.05%
F219	Influenza A virus (A/mallard/Alberta/356/2017(H4N6)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MH637264.1	97.34%

F220	Influenza A virus (A/Mallard/Ohio/18OS1894/2018(mixed)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT565512.1	96.14%
F221	Influenza A virus (A/American green-winged teal/Missouri/15OS6530/2015(H4N6)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KY583979.1	85.79%
F222	Influenza A virus (A/Hartlaubs gull/South Africa/18040224/2018(H5N8)) segment 7 matrix protein 1 (M1) gene, complete cds; and matrix protein 2 (M2) gene, partial cds	MN124659.1	97.56%
F223	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	99.45%
F224	Influenza A virus (A/blue-winged teal/LA/AI13-1437/2013(H7N7)) segment 7 matrix protein 1 (M1) and matrix protein 2 (M2) genes, partial cds	KJ413734.1	96.70%
F226	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	99.45%
F227	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	97.06%

F230	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	97.45%
F231	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	97.61%
F232	Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT624468.1	98.05%
F247	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.05%
F283	Influenza A virus (A/swine/Nigeria/IBDVR005/2015(H1N1)) segment 7 matrix protein 1 (M1) and matrix protein 2 (M2) genes, partial cds	KX429680.1	98.75%
F284	Influenza A virus (A/swine/Nebraska/A01774481/2016(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KX351170.1	98.75%
F287	Influenza A virus (A/Hawaii/24/2018(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MH245978.1	96.86%

F288	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	96.95%
F290	Influenza A virus (A/swine/Nebraska/A01774481/2016(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KX351170.1	99.38%
F291	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	96.59%
F292	Influenza A virus (A/swine/Nebraska/A01774481/2016(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KX351170.1	98.75%
F293	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	92.35%
F295	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	94.62%

F296	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	97.38%
F298	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	95.41%
F299	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	96.91%
F300	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	95.63%
F305	Influenza A virus (A/wild bird/Korea/L86-3/2008(H12N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	JX236023.1	98.01%
F306	Influenza A virus (A/Hawaii/24/2018(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MH245978.1	96.25%
F307	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes,	KU160677.1	97.77%

	complete cds		
F308	Influenza A virus (A/Hawaii/24/2018(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MH245978.1	94.55%
F310	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	97.91%
F311	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	96.08%
F313	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	95.28%
F314	Influenza A virus (A/swine/Arkansas/A01857979/2015(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	KU160677.1	96.61%
F315	Influenza A virus (A/shoveller duck/Shanghai/PD1018-32/2017(H6N5)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, partial cds	MH352246.1	96.61%

F316	Influenza A virus (A/Anas platyrhynchos/Belgium/11075_20/2017(H6N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT406874.1	96.97%
F324	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	99.45%
F364	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	96.14%
F365	Influenza A virus (A/mink/Eastern China/0712/2018(H5N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK849856.1	97.20%
F401	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	97.57%
F408	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.53%
F435	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.54%

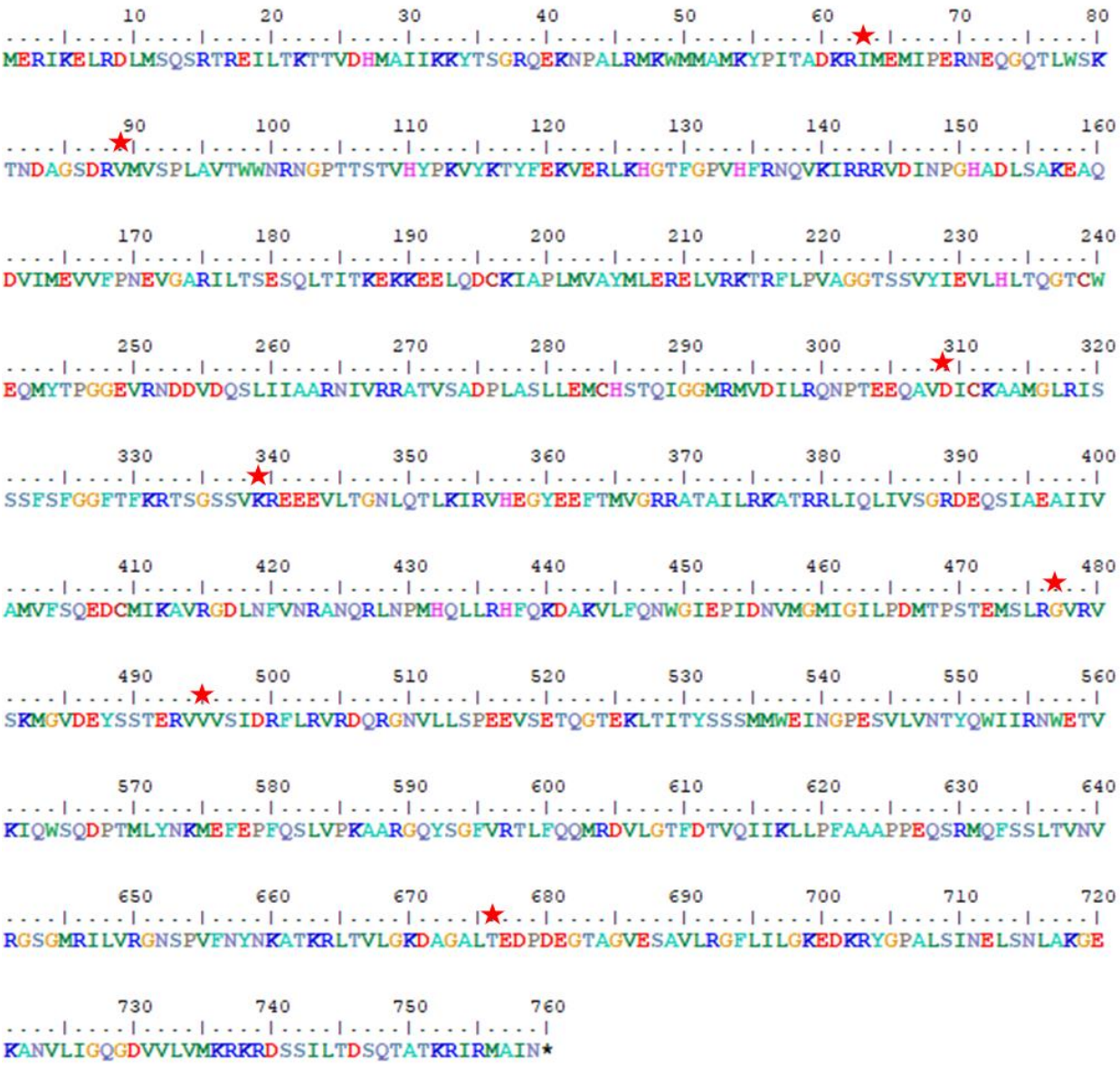
F436	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.53%
F437	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.04%
F438	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	97.60%
F439	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.54%
F440	Influenza A virus (A/Washington/9310/2019(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT638191.1	98.09%
F442	Influenza A virus (A/duck/Mongolia/667/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT020233.1	98.10%
F443	Influenza A virus (A/Iowa/70/2018(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MK475282.1	97.06%

F445	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	96.60%
F447	Influenza A virus (A/duck/Mongolia/667/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT020233.1	97.61%
F457	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.04%
F483	Influenza A virus (A/duck/Mongolia/667/2019(H3N8)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	MT020233.1	98.53%
F491	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.95%
F492	Influenza A virus (A/mallard/Sweden/3269/2003(H7N7)) matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds	CY184577.1	98.48%

APPENDIX 3. Amino acid sequences of gene products of GD/18 and amino acid markers

(GenBank accession numbers: MT783429-to-MT783436 in the order of PB2, PB1, PA, HA, NP, NA, M and NS)

PB2



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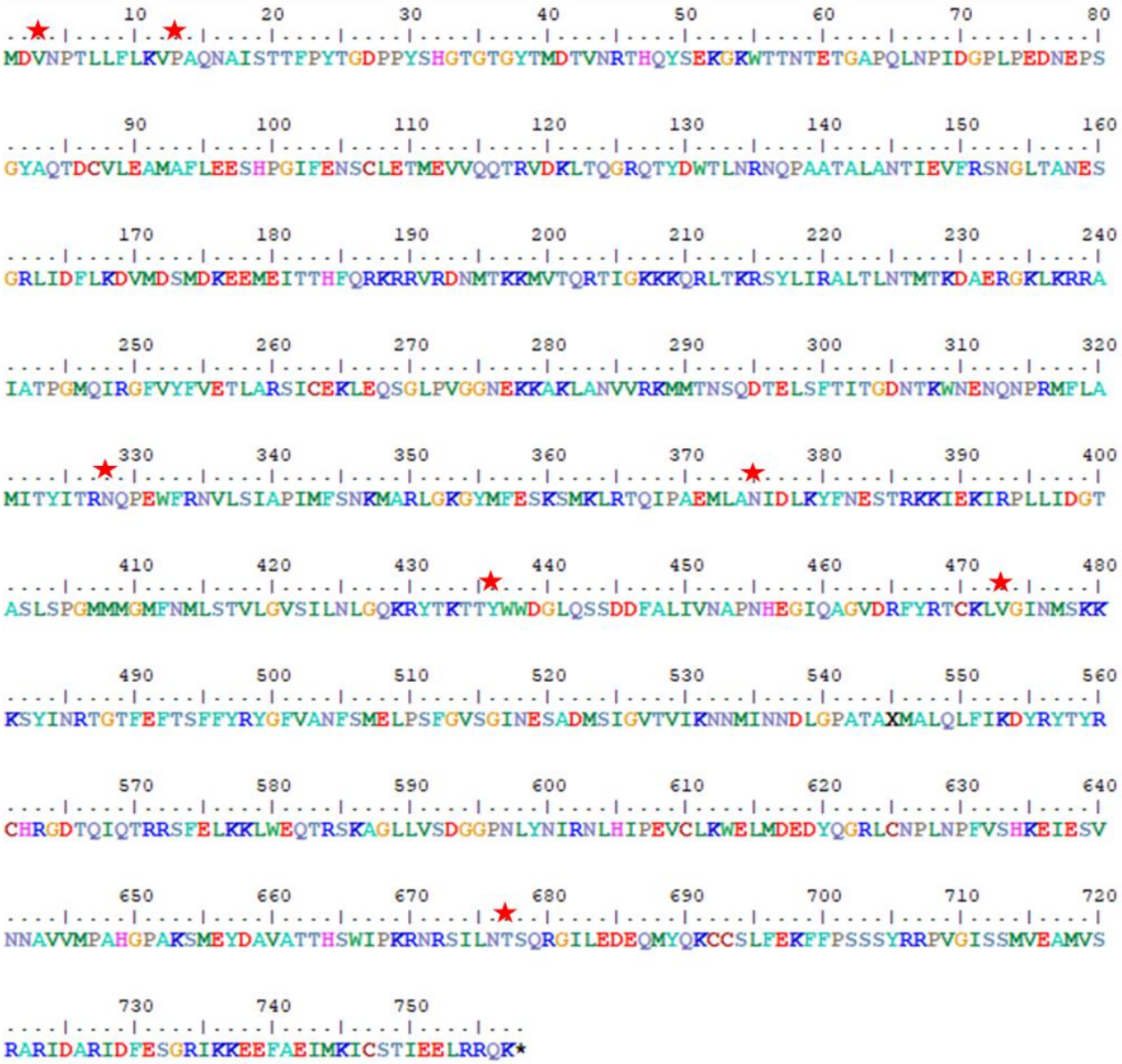
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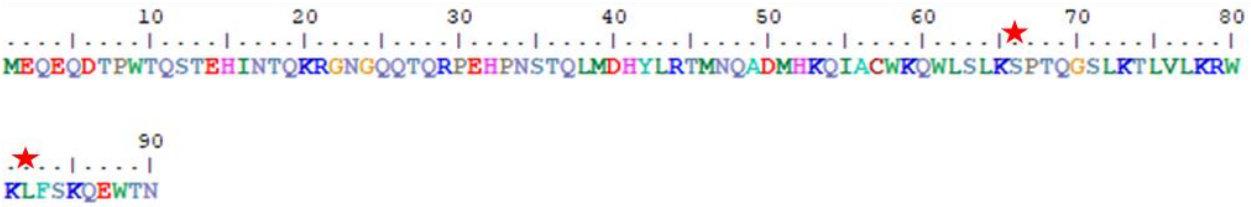
PB1-F2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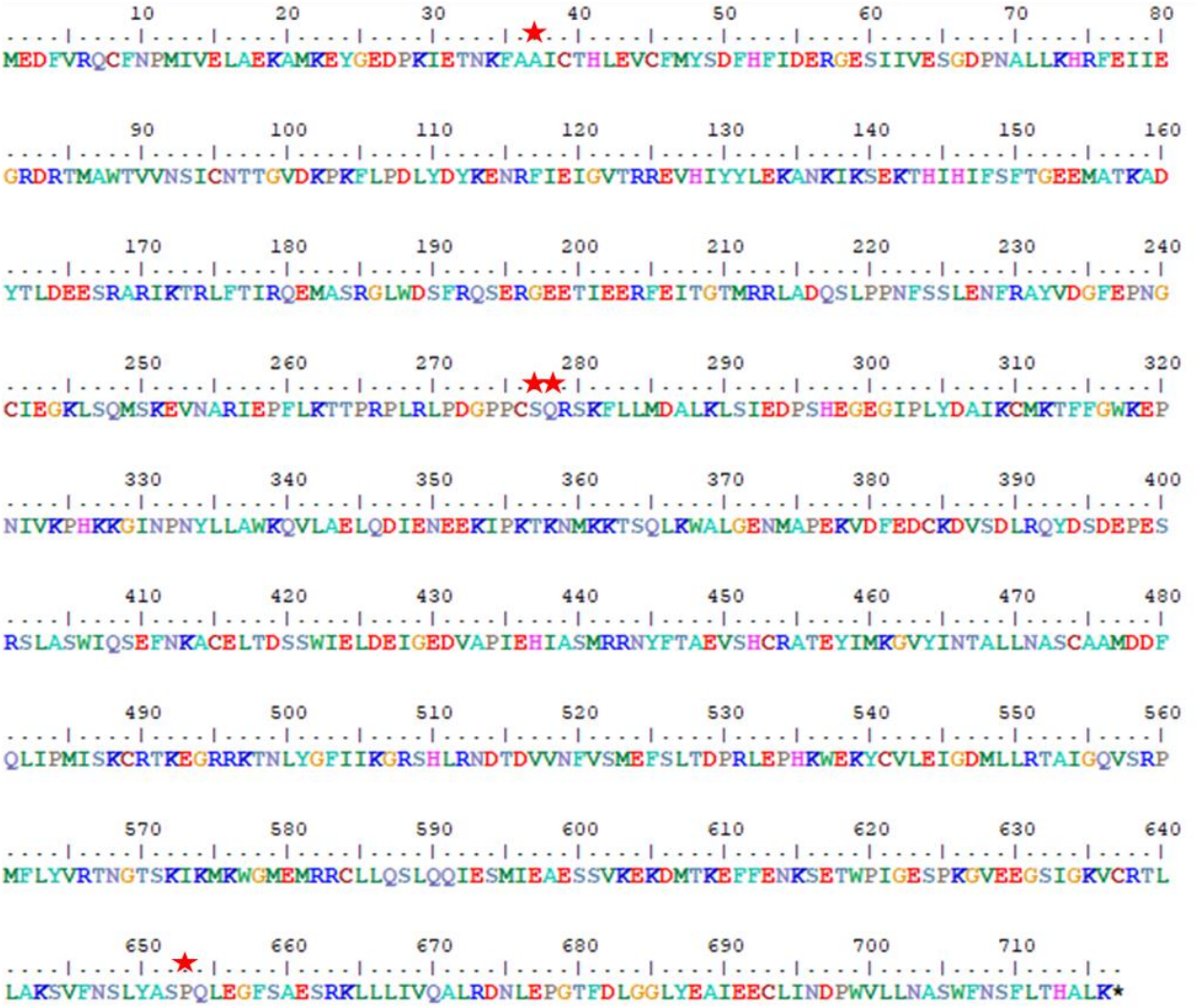
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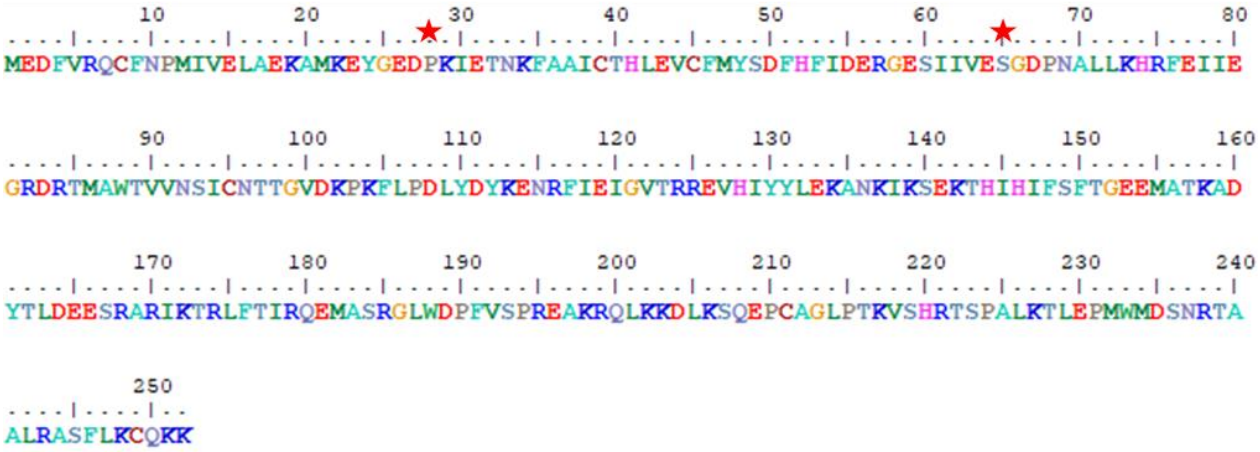
PA-X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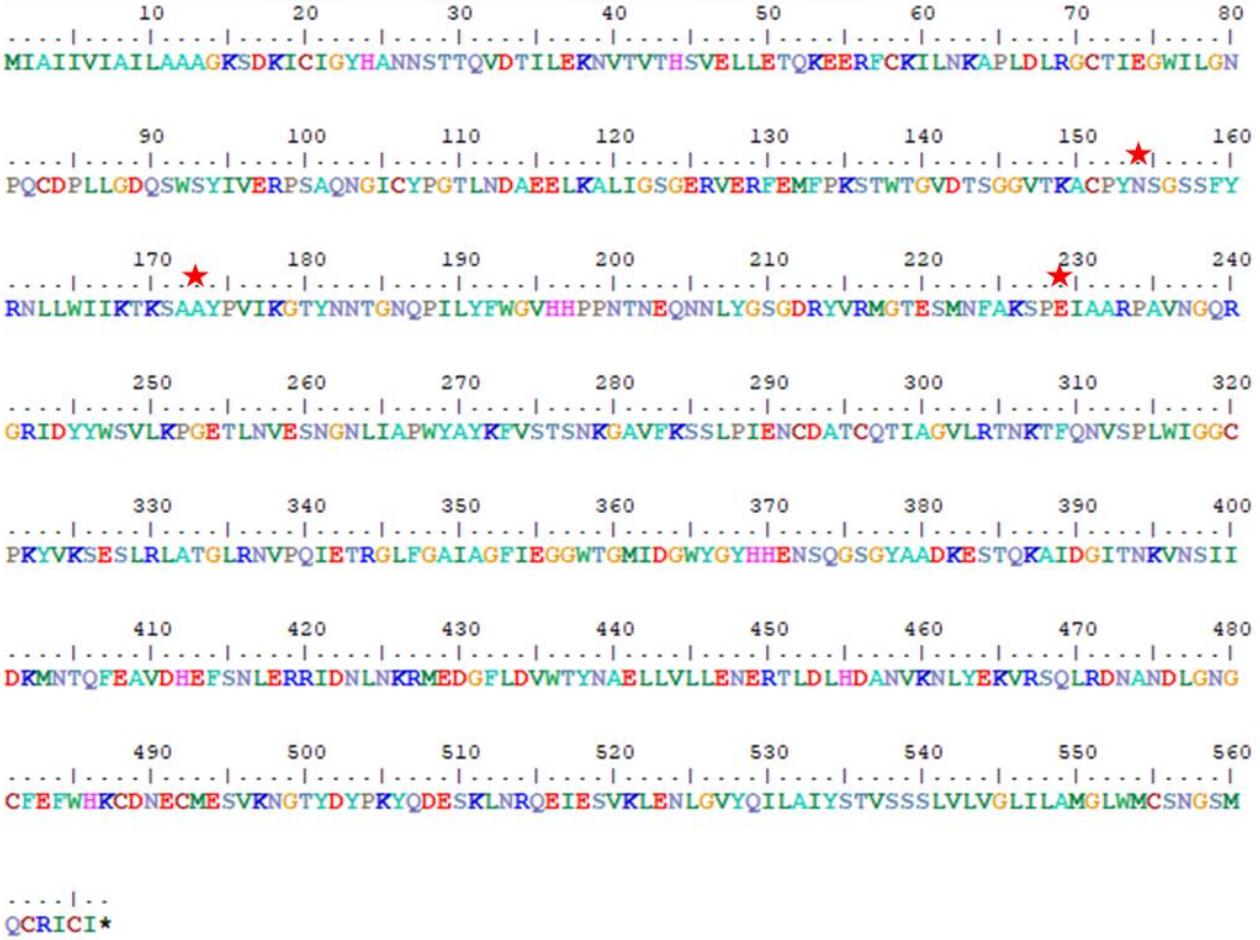
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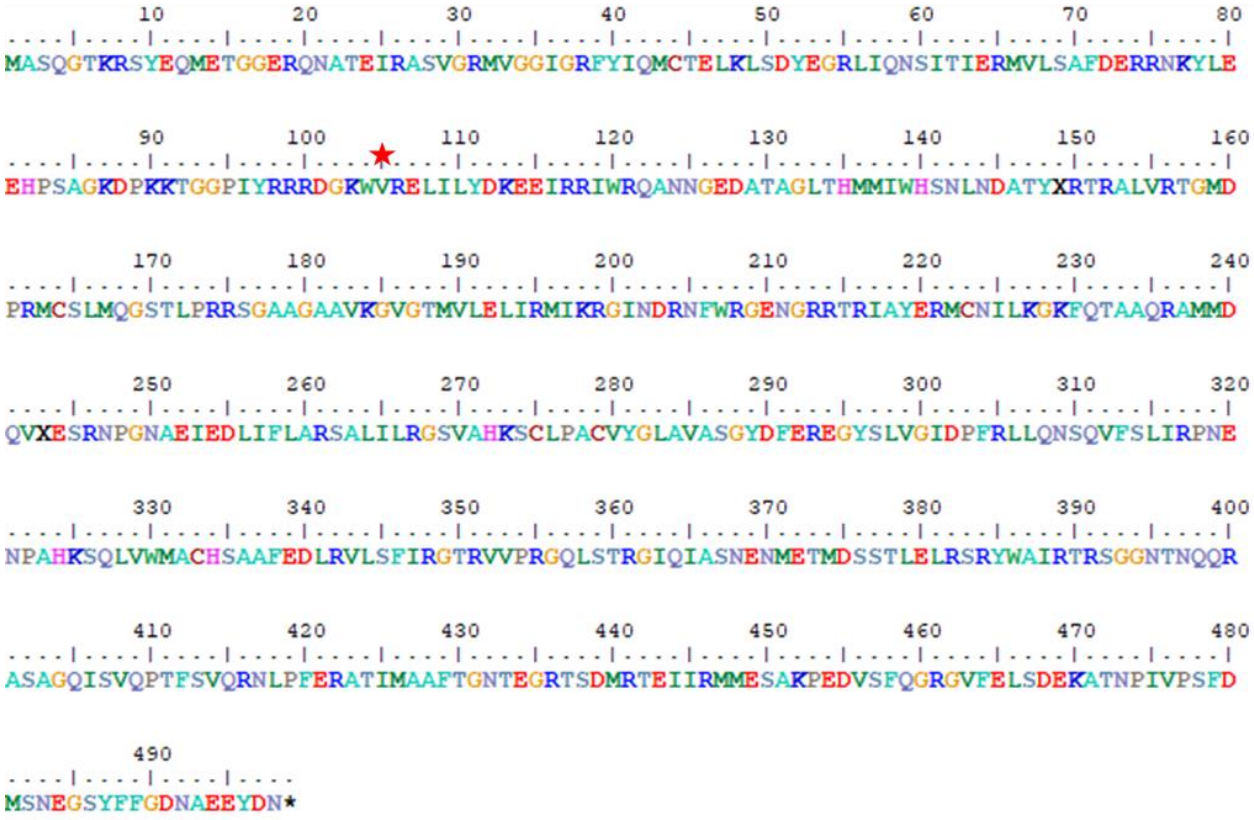
NP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490
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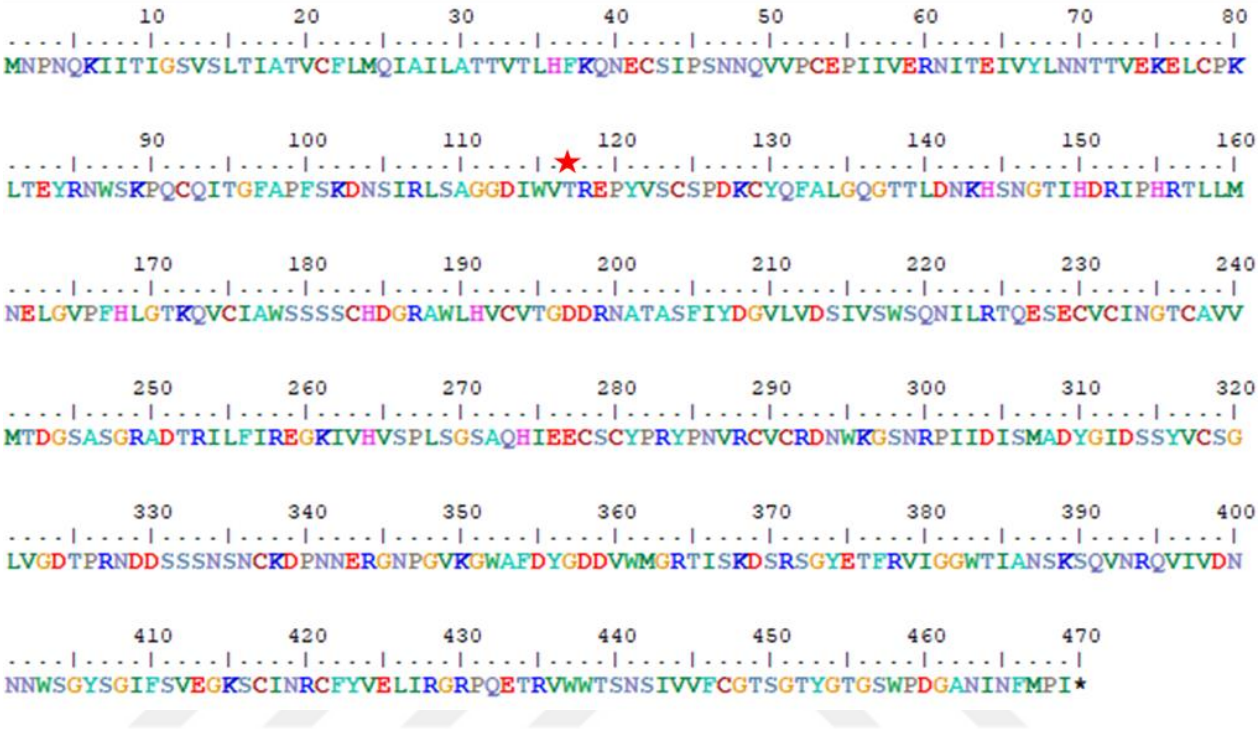
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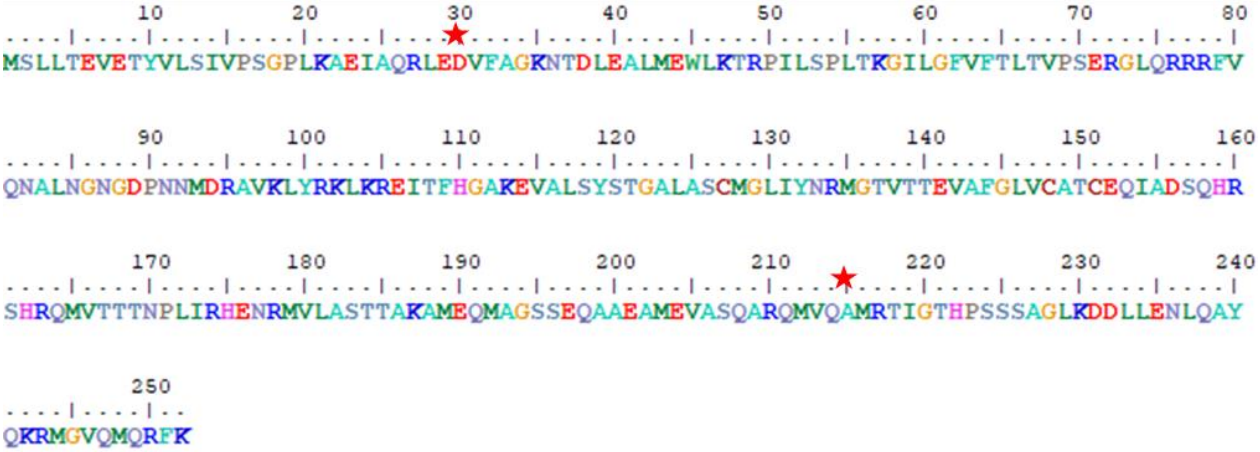
M1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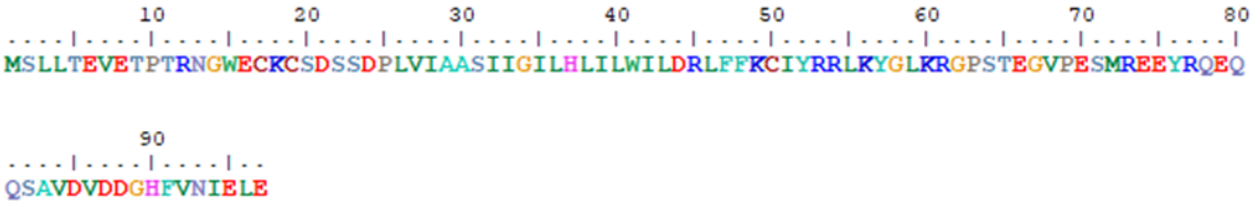
M2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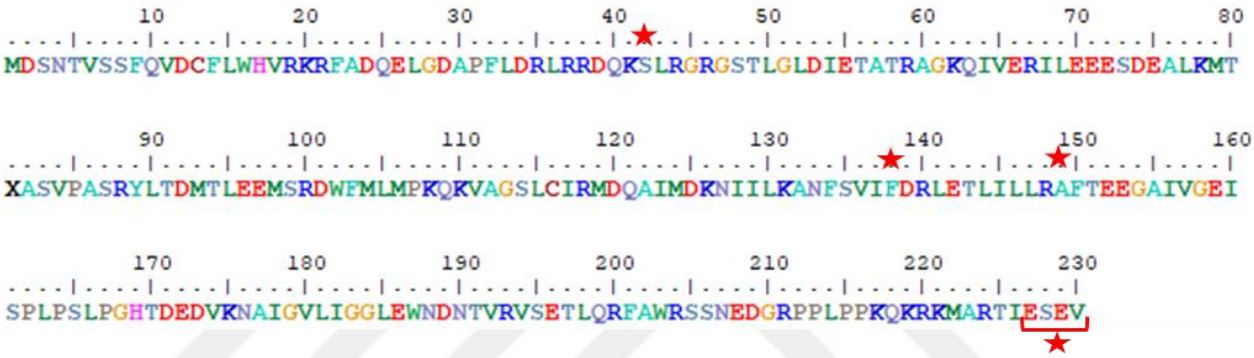
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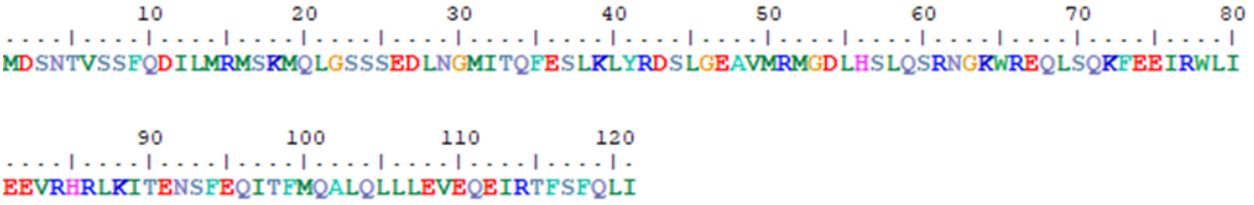
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8. ADENDA

8.1. Curriculum Vitae Yavuz MERCAN

PERSONAL INFORMATION

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EDUCATION

Years: 2011 – 2016

School: Izmir Institute of Technology

Department: Molecular Biology and Genetics, Bachelors

School: Göteborgs Universitet – Göteborg Üniversitesi / İsveç **(Erasmus+, january – June 2015)**

Department: Molecular Biology and Genetics

Years: 2016 – 2017

School: Izmir Institute of Technology,

Department: Molecular Biology and Genetics, Master's

Years: 2017 – present

School: Dokuz Eylül University, Izmir - IBG (Izmir International Biomedicine and Genome Institute),

Department: Molecular Biology and Genetics, Master's

RESEARCH EXPERIENCE

- Molecular Medicine Laboratory (July, 2013 – June 2016)
  Molecular Biology and Genetics / Izmir Institute of Technology

  Supervisor: Prof. Dr. Volkan SEYRANTEPE

  Research Topic: The Role of Lysosomal Enzyme Cathepsin A on Vasoactive Peptide Biology

- Molecular Virology Laboratory (June, 2016 - September, 2016)
  Department of Microbiology / University of Padua

  Supervisor: Assistant Proffessor Gualtiero Alvisi

  Research Topic: Generation of Stable Cell Lines Expressing Fluorescent Signal in Case Of Hepatitis C Virus Infection.

- Molecular Microbiology Laboratory (September 2016 – September 2017)
  Molecular Biology and Genetics / Izmir Institute of Technology

  Supervisor: Associate Professor Alper ARSLANOĞLU

  Research Topic: Investigation of the Production of SLPI Protein in the Presence of HIV-1 Tat in African Green Monkey CV-1 Cell Lines and Effects on HIV-1 LTR

- Koçer Lab. August, 2017 – present
  IBG–Izmir (Izmir International Biomedicine and Genome Institute)
  Supervisor: Assistant Professor Zeynep Ahsen Koçer
  Research Topic: Prevalence and Molecular Characterization of Low Pathogenic Influenza Virus from Wild Waterfowls and Shorebirds in Gediz Delta, Izmir/TURKEY

PRESENTATIONS

• Molecular Characterization and Isolation of Influenza A Viruses from Wild Aquatic Birds in Gediz

Delta, EEBST2019

REWARDS

• Best Poster Presentation Award 6th EEBST (Ecology and Evolutionary Biology Symposium)

CERTIFICATES

• Certificate of Experimental Animal Use, Dokuz Eylül University Local Ethical Committee of Animal Experiments, 2016

REFERENCES

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