Avian Bird Influenza type A viruses : Current situation in Europe, epidemiological considerations, prevention, treatment and Biosafety measures

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Abstract

This dissertation was written as part of the MSc in Bioeconomy: Biotechnology and Law at the International Hellenic University.
Avian (bird) Influenza Type A Viruses (AIVs), occur naturally among wild aquatic birds worldwide and might infect domestic poultry and other birds and animal species. Avian influenza viruses don't normally infect humans, but sporadic human infections have occurred. At least 16 subtypes of the viral heamagglutinin (HA) glycoprotein and 9 of the viral neuraminidase (NA) are distinguished as a result of natural evolution.
AIVs vary in their ability to produce infection, disease, and death in different bird species. Based on the pathological effect they are categorized as low pathogenic (LPAIV) and highly pathogenic (HPAIV) viruses. Infection with LPAIV causes asymptomatic infections in wild aquatic birds. Poultry infections with LPAIVs may be asymptomatic or may only cause mild symptoms (ruffled feathers and drop egg production) or typically produce respiratory, digestive, and reproductive signs. The HPAIVs have been seen primary in gallinaceous poultry, producing high morbidity and mortality and systemic disease with necrosis and inflammation in organs, nervous and cardiovascular systems.

Although HPAIV strains seldom infect domestic waterfowl or wild birds, the Eurasian African H5N1 HPAIV has evolved over the past decade with the unique capability to cause infection in domestic ducks and wild birds. Indeed, HPAIV show zoonotic characteristics and can be transmitted from birds to different mammalian species including humans. The economic aspect of infected poultry is tremendous.

Theoretically, pandemic viruses might derive directly from AIV or after genetic reassortment between viruses of avian and mammalian origin. Fighting the H5N1 at its source is the prerequisite to reduce pandemic risks posed by this virus. Other influenza viruses regarded as pandemic candidates derive from subtypes H2, H7 and H9 and have infected humans in the past.

The aim of the dissertation is the literature review of the epidemiological status of the Avian influenza virus in Europe with focus on the situation in Greece, presenting data from the last decade concerning the outbreaks and the surveillance programs according to the EU legal framework.

First I would like to express my special gratitude to my supervisor Dr. Savvas Genitsaris for his valuable advice, motivation and support during this Msc. Last but not least, I would like to thank my family especially my husband Georg for supporting me spiritually throughout writing this dissertation.

Keywords: (Europe, Avian influenza virus, prevention, control, Greece)

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Preface

The first cases of Avian influenza A in birds (HPAI virus H5N1) in Greece, was reported in January 2006. The HPAI outbreaks occurred in six different regions of the country (Central Macedonia, east Macedonia and Thrace, West Macedonia, Central Greece, Peloponnese, and Aegean Islands). Totally 16 swans (*Cycnus olor*) and one red-breasted wild goose (*Branta ruficollis*) were found dead. A new strain of HPAI virus (HPAI H5N8) was also identified in 2016, which was the first case of dead in wild bird species and poultry in Greece.

Contents

1. Introduction

1.1. The poultry sector in the E.U

In Europe, the production of poultry has been doubled between 2000 and 2018, while the common European wild birds' populations have declined (EBCC/RSPB/BIRDLIFE/CSO, 2020). Poultry represents a significant source of high-quality animal protein of relative low cost; because of the rapid growth rate and the adequate conversion of feed into body weight consumers consider the poultry meat as a cheap and healthy sustainable source of food (Otekunrin, 2018). Therefore, as the worldwide demands for meat consumption are continually increasing, following a raising world population, an increase in the production of poultry meat is projected. The production of meat is set to adapt to sustainability objectives like increasing carbon sequestration, the production in non-conventional systems such as organic etc. Since sustainability is a major concern nowadays, it's expected to drive meat markets over the coming decade, by pushing for lower consumption per capita, more efficient production systems with fewer animals and even reduced exports of animals (OECD/FAO 2021).

Chicken consumption ranks in second place (35.2 %) after pork meat consumption (37 %) of animal origin intended for human consumption globally. The United States of America holds the largest position in poultry meat production, with 17 % of global output, following by China and Brazil. The world's leading producer is Asia representing 44 % of global poultry production, while China alone meets about 37 % of the global egg producer in the world, followed by United States 7 % and India by 6 %. In 2019, poultry meat production represented 39 % of the global production in meat and the world egg production has increased around 150 % in the last decades. Overall, in the EU the meat consumption per capita is projected to decline from 69.8 kg in 2018 reaching 67.0 kg by 2031 (ec.europa.eu).

Poultry is the only meat category according to the EU agricultural outlook report for 2021-2031, whose production has expanded during the COVID-19 pandemic and is the only one await to multiply during 2020 and 2030 (ec.europa.eu). Also, the consumption is expected to reach 24.8 kg per capita by 2031 (+1.3 kg compared to 2020) and the demand is as well expected to increase in key exports destinations. The pig meat in the EU is expected to fall by 0.5 % per year from 32.5 kg in 2021 to 31 Kg in 2031 per capita. This phenomenon is related to the impact of African Swine Fever in Asia on the EU meat market. However EU is expected to remain a leader supplier of pig meat to the global market. Following a decrease in the EU total cow herd, the production in beef meat is expected to continue downwards between 2021 and 2031 (from 10.6 kg to 9.7 kg) (ec.europa.eu) (fao.org).

In 2020, the main poultry meat production in the EU (Figure 1) includes the key producers Poland (2.7 million tonnes about 19.8 %), Spain (1.7 million tonnes about 12.6 %) France (1.7 million tonnes about 12.3 %) Germany (1.6 million tonnes about 11.9 %) and Italy (1.4 million tonnes about 10.2 %) in a total of 13.6 million tonnes of poultry meat production in 2020 in EU (Eurostat, 2020).

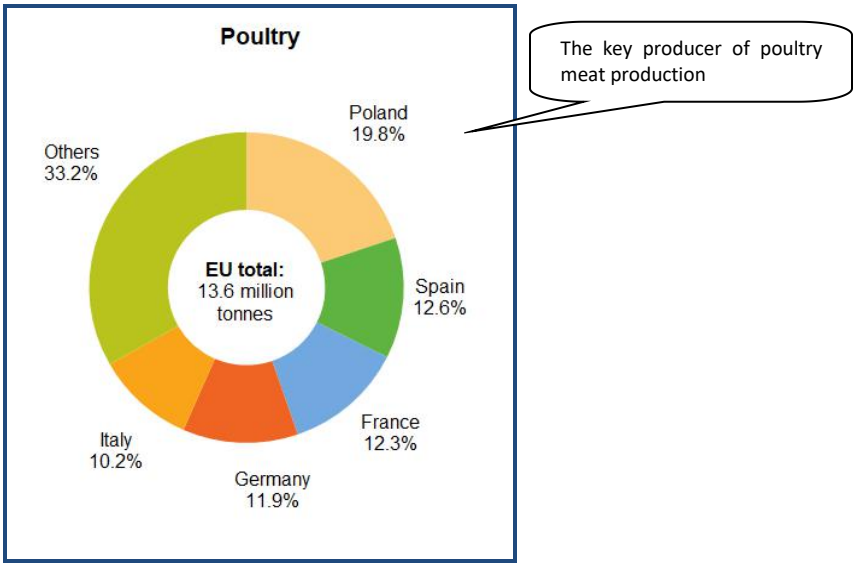

Figure 1: Share of quantity of EU Poultry meat production. Modified by Source: Eurostat 2020.

1.2 Avian Influenza Virus (AIV) transmission and infection: An overview

Avian influenza (AI) is known as the disease caused by infection with avian bird influenza Type A viruses (AIVs). It remains complex in the epidemiology occurring naturally among wild aquatic birds worldwide and might infect poultry and other birds and animal species. AIV strains may affect a wide range of hosts including mammals (humans, pigs, horses, seals, whales, mink, dogs, felids, and civets) and may therefore pose a zoonotic risk. Indeed, sporadic infections with avian flu viruses have occurred, even if AIVs do not normally infect humans.

Wild birds, especially those belonging to the orders Anseriformes (swan, ducks, geese, and waterfowl) and Charadriiformes (gulls, terns, and shorebirds) are recognized as the natural reservoirs and hosts for the low pathogenic avian influenza (LPAI) viruses. These LPAIVs are isolated so far from over than 100 wild bird species (25 families) which shows the LPAIVs have a global distribution among free living aquatic avian populations. It is recognized that LPAIVs are often transmitted directly or indirectly to domestic birds, wild or domestic animals and even humans. The exact route of the source and contagion

of the virus and the different genes among avian and mammalian species are demonstrated in Figure 2 (Joseph et.al, 2016).

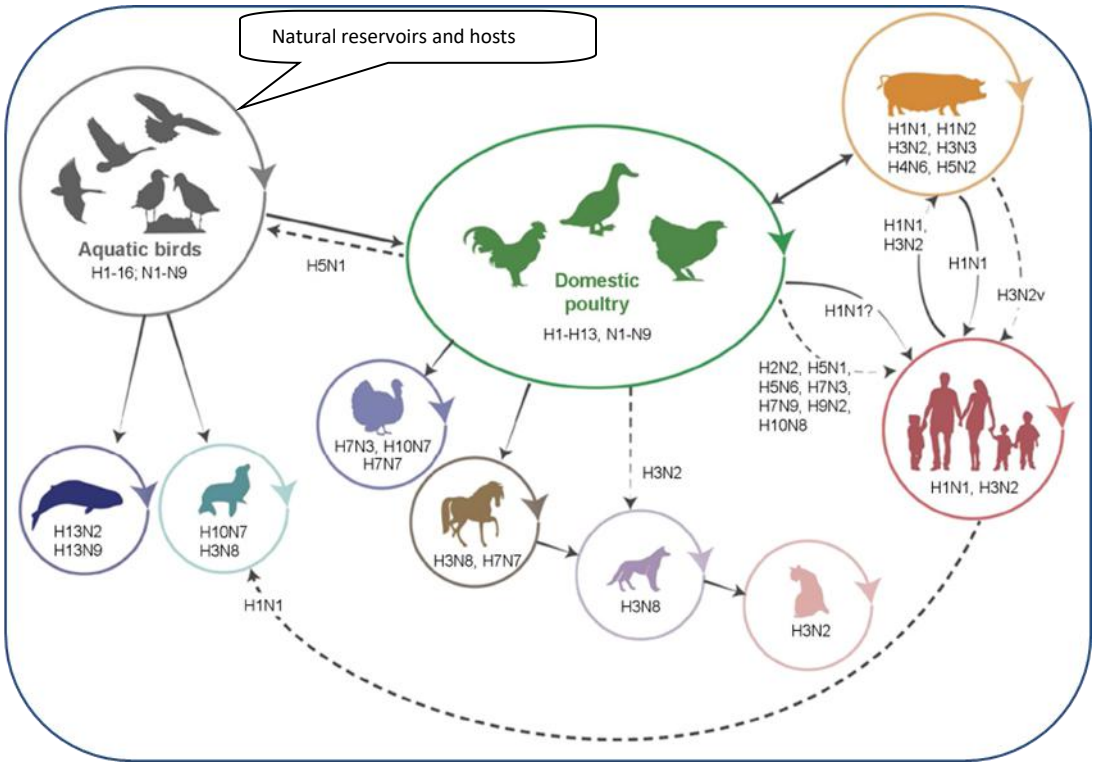

Figure 2: Schematic of transmission of AVIs modified by source Joseph et.al, 2016. Influenza and Other Respiratory viruses. Wiley, 2017

On the other hand, highly pathogenic avian influenza (HPAI) viruses are not normally present as host reservoirs in wild birds. But the LPAIVs of type H5 and H7 can evolve into HPAIVs upon introduction into poultry causing serious mortality and disease particularly in the order Galliformes (chicken and turkeys). From the poultry the virus can be transmitted to wild bird's population (Figure 2) circulating in asymptomatic individuals or causing disease and mortality (spillback phenomenon) (Lee, 2017) (Shriner S, Root J., 2020).

The epidemiology of viruses and their evolution is a consequence of the interaction between the viruses, their hosts, and their environments. An important component of the virus epidemiology and evolution occurs at the wild-domestic interface, which can introduce the virus into the flocks. Sharing poultry equipment, people fomites and carcass not proper disposed might contribute to the transmission. In some cases, it is also described because of direct contact between infected and susceptible bird species, via faeces, since the viral load is very high there, as well through other secretions (respiratory, saliva, nasal) (Bowes et al., 2004).

The main source of the virus remains faeces, nasal, respiratory secretions, and saliva. For many years the predominant way of spread in wild birds was thought to be the excreted faeces and the faecal-oral way. Recently, isolates of HPAIV H5N1 were found in major quantities in tracheal samples compared to faecal samples (Fields et al., 2007). This could indicate that the spread of the virus happens via respiratory secretions, in specific subtypes, like HPAIV H5N1. However, higher quantities of other HPAIVs are isolated from the tissues of infected birds. This indicates the viral titers vary depending on the strain, host, and the clinical stage of the infection.

All the equipment used in poultry holdings and people's personal equipment (shoes, clothes etc.), water egg crates etc. are consider as fomites of the virus. Moreover, broken eggs can transfer the virus in poultry via the incubator. Isolation of HPAIVs in the eggshell and inner egg contents has been made, but vertical transmission could not be verified, since the most of the strains are embryo-lethal and thus eggs are not able to hutch (Mak et al., 2012).

The degree of host adaptation diversifies by how close the hosts are genetically, and interspecies transmission has been noted between species that are not so close related (different order), like the transmission from species belonging to the Anseriformes order to Galliformes order species (Swayne, 2000) (Joseph et. al, 2016). Direct transmission between different phylogenetic classes, like chicken to humans, is counted very unlikely. However, synergically factors like geographic proximity, the presence of high bird density, low temperatures etc. may increase cross species transmission as a result the increase opportunities for exposure and expedite the virus viability in the environment. Truly, this began in 1997 when an H5N1 virus was transmitted directly from poultry to man, after direct contact with sick birds (Modena, 2007) (Charisis, 2008). According to WHO HPAIV A (H5N1) has caused 862 human cases worldwide as presented in Table 1, including 455 deaths between 2003-2021. In Europe no human cases have been reported so far and these viruses lack the capability for sustained human to human transmission. During the last decade there have been reports of human infections with subtype H1N1 and H3N2 from pigs, so the risk exists, even if it is assumed to be low. Nevertheless, AIVs pose a pandemic threat, producing new viral strains-subtypes, due strategies that lead to mutation in single point of the genome which can alter HA or NA structure, resulting in a different strain (antigenic drift) or due reassortment of segments which can affect the HA, NA, or other proteins (antigenic shift) (Horimoto, 2005) (Tarek et al. 2021).

The ability of the virus to undergo variation due reassortment and mutations clearly shows the possibility of adaptation to humans, although H5N1 has not been capable of human-to-human transmission. Therefore, the WHO appeals for an pandemic alert, that involves not only be prepared for an influenza pandemic, awareness to stop initial outbreaks, means case recognition, sensitive rapid diagnostic methods, and preventive measures to reduce spread of the disease (WHO, 2021).

Table 1: Cumulative number of confirmed human cases for avian influenza A (H5N1) worldwide between 2003-2021. Modified by source WHO, 2022

COUNTRY	2003-2009		2010-2014		2015-2019		2020		2021		TOTAL	
	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS	CASES	DEATHS
AZERBAIJAN	8	5	0	0	0	0	0	0	0	0	8	5
BANGLADESH	1	0	6	1	1	0	0	0	0	0	8	1
CAMBODIA	9	7	47	30	0	0	0	0	0	0	56	37
CANADA	0	0	1	1	0	0	0	0	0	0	1	1
CHINA	38	25	9	5	6	1	0	0	0	0	53	31
DJIBOUTI	1	0	0	0	0	0	0	0	0	0	1	0
EGYPT	90	27	120	50	149	43	0	0	0	0	359	120
INDONESIA	162	134	36	31	3	3	0	0	0	0	200	168
IRAK	3	2	0	0	0	0	0	0	0	0	3	2
LAO PEOPLE'S DEMOCRATIC REPUBLIC	2	2	0	0	0	0	1	0	0	0	3	2
MYANMAR	1	0	0	0	0	0	0	0	0	0	1	0
NEPAL	0	0	0	0	1	1	0	0	0	0	1	1
NIGERIA	1	1	0	0	0	0	0	0	0	0	1	1
PAKISTAN	3	1	0	0	0	0	0	0	0	0	3	1
THAILAND	25	17	0	0	0	0	0	0	0	0	25	17
TURKEY	12	4	0	0	0	0	0	0	0	0	12	4
VIETNAM	112	57	15	7	0	0	0	0	0	0	127	54
TOTAL	**468**	**282**	**233**	**125**	**160**	**48**	**1**	**0**	**0**	**0**	**862**	**455**

1.3. History of AI – Outbreaks in EU

Today it has been established that flu pandemics are likely to appear every 10-15 years, with the emergence of new virus that spread rapidly all over the world's population. In 1918, the deadliest influenza pandemic in human history was the Spanish flu caused by an H1N1 virus apparently of avian origin. It is estimated that about 500 million people became infected, and the number of deaths was about 50 million people worldwide (cdc.gov).

The first cases of HPAI termed as fowl plaque, were reported in 1878 in chicken in Italy, followed by geographically dispersed outbreaks through the late 1800s to the 1950s in Europe, Asia, America, and Africa. Since 1959, many outbreaks have been reported and

the HPAIVs arose from H5 and H7 LPAIVs after circulation for weeks or years in gallinaceous poultry, developing specific mutations in the HA gene, that lead to high pathogenicity of the virus. The most epizootics have been limited in farm-to-farm spread in poultry and were eradicated by stamping out methods. However, in 1996 an avian influenza virus H5N1 emerged in Guangdong China, in domestic goose and to date, this lineage (Gs/GD lineage) caused deaths in poultry, but also wild birds and humans. The virus spread to over 80 countries in Asia, Europe, America, and Africa, changing the perspective on HPAIV biology (Lee et al., 2017) (Verhagen et al., 2021) (Table 2).

Table 2: Influenza Pandemic in Human History

PANDEMIC INFLUENZA HUMAN	YEAR	SUBTYPE
RUSIAN INFLUENZA	1889	H2N2
OLD HONG KONG FLU	1900	H3N8
SPANISH FLU	1918-1919	H1N1
ASIA FLU	1957-1958	H2N2
HONG KONG FLU	1968-1969	H3N2
PANDEMIC INFLUENZA	2008	H1N1

Since 2008, HPAIV subtypes H5N8, H5N5, H5N2 bearing the backbone of Gs/GD lineage H5 clade 2.3.4.4 have been identified in China in domestic ducks and poultry in live markets. Scientists pointed out that the clade 2.3.4.4. HPAIVs have undergone genetic reassortment with other clades of H5N1 viruses and with local LPAIVs (Guo et al., 2021).

After 2014, HPAIV H5 clade 2.3.4.4 viruses have spread very fast, globally (from East Asia to North America, west Asia, and Europe) through wild birds and reassortment with prevailing LPAI viruses. Permissive to infection and transmission of clade 2.3.4.4 viruses are wild and domestic waterfowl, domestic poultry, and zoo birds. Several studies pointed out that HPAIV infection with H5 reassortment show reduce virulence birds compared to Gs/GD H5N1 virus. It is interesting that clade 2.3.4.4 viruses in waterfowl act as asymptomatic spreaders, being able to spread unrecognized explaining the globalization of this lineage and the potential for virus dissemination by intercontinental wild bird migration (Lee, 2017). Even H5N8 HPAIV was first reported in the period 2014-2015 in the EU, it was not associated with die-offs of wild birds. During that time a small number of wild birds were tested positive during active surveillance in Germany and the Netherlands. The virus had close sequence homology to Asian H5N8 HPAIV strains detected in China and South Korea in 2014 and was highly pathogenic for chickens and mildly for wild ducks (Bouwstra et al, 2015) (Alarcon et al. 2018).

Highly pathogenic avian influenza H5N6, a related virus that is also in the clade 2.3.4.4 that was previous found in China, Vietnam, and Laos, was found for the first time in Republic of Korea and Japan from dead wild birds, captive animals in Japan and poultry

only in Korea in 2016. It is important to note that this group of viruses has been associated with human infection, including several deaths (Lee et al, 2017).

The 2016-2017 epizootic was the largest in Europe by number of farms and countries affected and the greatest diversity of wild birds infected. Scientists observed significant differences among the three epizootics (2005-2006, 2014-2015, 2016-2017) regarding the region affected, epidemic curve, the season and outbreak duration, making it hard to predict a future HPAIV epizootics (Alarcon et al., 2018).

The largest epidemic recorded so far in Europe was between October 2016 and March 2017 (Figure 3). Almost all countries reported outbreaks in poultry and a high number of infected dead wild birds. The virus spread mainly from East to South-West and further to Africa affecting countries in the western and southern part of the continent. Migratory wild birds were implicated as the vector delivering HPAIV H5N8, clade 2.3.4.4 across the European continent. However, wild birds were an important component in the spread of HPAIV to the commercial poultry sector and significant secondary spread between poultry holdings was also seen in a number of European countries, especially Bulgaria, France, Hungary and Germany (Alarcon et al., 2018, Grund, 2019).

H5 HPAIVs are continuing to cause outbreaks in poultry and wild birds globally, with devastating economic consequences. France was massively affected in 2016-2017 with extensive farm to farm spread, predominantly in ducks. This leads to serious impact on poultry production and trade, as well as on farmers live hood (400 farms affected). In Hungary more than 200 farms were infected. Italy was also heavily affected with 2.7 million domestic birds across 83 farms.

On December 2017, a case of H5N6 HPAIV in a commercial duck holding in Netherland, followed later by 2 cases in captive birds, and in four mute swans (*Cycnus olor)* was reported. The national reference laboratory for avian influenza characterised the virus as a reassortment of the 2016 H5N8 HPAIV. The virus contained a novel N6 gene segment from a European LPAIV, and the virus is similar to the virus detected in Greece in 2017 (Alarcon et al., 2018).

During 2018-2019 in Europe, only 21 outbreaks in two countries, Bulgaria and were reported. Two subtypes were detected in the poultry outbreaks in Bulgaria, HPAIVs H5N8 and H5N2. The analyses produced by the reference labour of the countries showed that H5N2 and H5N8 viruses are co-circulating in Bulgaria and are not related to the HPAIV H5N8 viruses circulating currently in other European countries (efsa.europa.eu/efsajournal, 2020) (Table 3).

Table 3: HPAIV outbreaks in China and Europe between 1996 - 2022

HPAIV POYLTRY AND NON POYLTRY COUNTRY	YEAR	SUBTYPE
CHINA	1996	H5N1
	2008	H5N8 H5N5 H5N2
EUROPE	2014-15	H5N8
	2016-2017	H5N8 H5N6
	2018-2019	H5N8 H5N2
	2020	H5N1 H5N8
	2021	H5N8
	2022	H5N1

In Europe in 2020, 561 HPAIV (H5) outbreaks in poultry, captive and wild birds were reported. Most cases were in wild birds (510) predominantly in Germany, Denmark, and the Netherlands; 43 outbreaks in poultry and 8 outbreaks in captive birds. Also, three LPAIV outbreaks were reported in Germany, Italy, and the UK. Analyses of the gene segments identified four distinct genotypes one H5N8, one H5N1, and two H5N5, originating from multiple reassortment events with LPAIVs circulating in wild birds in Eurasia. Specifically, the H5N8 genotype had the same gene constellation of the H5N8 virus introduced into Europe during 2016-2017. The H5N1 shares the same HA and M genes with H5N8, while the remaining gene segments have been acquired through reassortment with LPAIVs circulating in wild birds in Eurasia. This strain is not related to H5N1 viruses circulating in East Asia that caused human infections. The two H5N5 viruses identified belong to two different genotypes (efsa.europa.eu/efsajournal, 2020) (Figure 4).

In 2021, outbreaks of HPAI H5N8 were confirmed in poultry and wild birds in different countries in Europe and especial the spring 2021 HPAI epidemic was the largest recorded in Europe. The first cases were detected in turkey holdings, and later in layers, broilers, backyard flocks and wild birds, as well as breeder flocks. Until June 2021, there were more than 1200 cases in poultry involving 22.4 million poultry birds in 27 different EU countries reported predominantly in Poland Germany and the Netherlands. Most cases were in wild birds (1665) predominantly in Germany and Denmark. Analyses of the gene segments identified two distinct genotypes (one H5N8, one H5N1) originating from multiple reassortment events with LPAIVs circulating in wild birds in Eurasia. Specifically, the H5N8 genotype had the same gene constellation of the H5N8 virus introduced into Europe during 2016-2017 (efsa.europa.eu/efsajournal, 2021) (Figure 4).

So far on this year (February 2022) 16 European states reported HPAI outbreaks in poultry and wild birds linked to the subtype H5N1. The most affected by the spread of infection was France were 415,000 poultry mostly flocks of ducks, but also chicken broilers, turkey, quinea fowl and mixed species were involved in the outbreaks.
In Spain almost 319,000 poultry (turkey, laying hens and breeder hens) have been impacted. Outbreaks in Portugal involved 124, 500 poultry. Also Hungary reported 29

outbreaks followed by Poland (21), Italy (16) and Germany (15) until February 2022. As far as wild birds concerned almost 1000 HPAI outbreaks in Europe have been reported until February with the most cases in Germany (494) followed by the Netherlands (185) and Denmark (55). In all cases again the H5N1 subtype was confirmed.

In recent years, several outbreaks have been reported in different parts of Europe, Asia, and Africa, raising concerns about dissemination of new lethal influenza pandemic. Although H5N1 is not capable of sustaining human to human transmission, the ability of the virus to undergo variation due mutations and reassortment, it poses the possibility of viral adaptation to the human species.

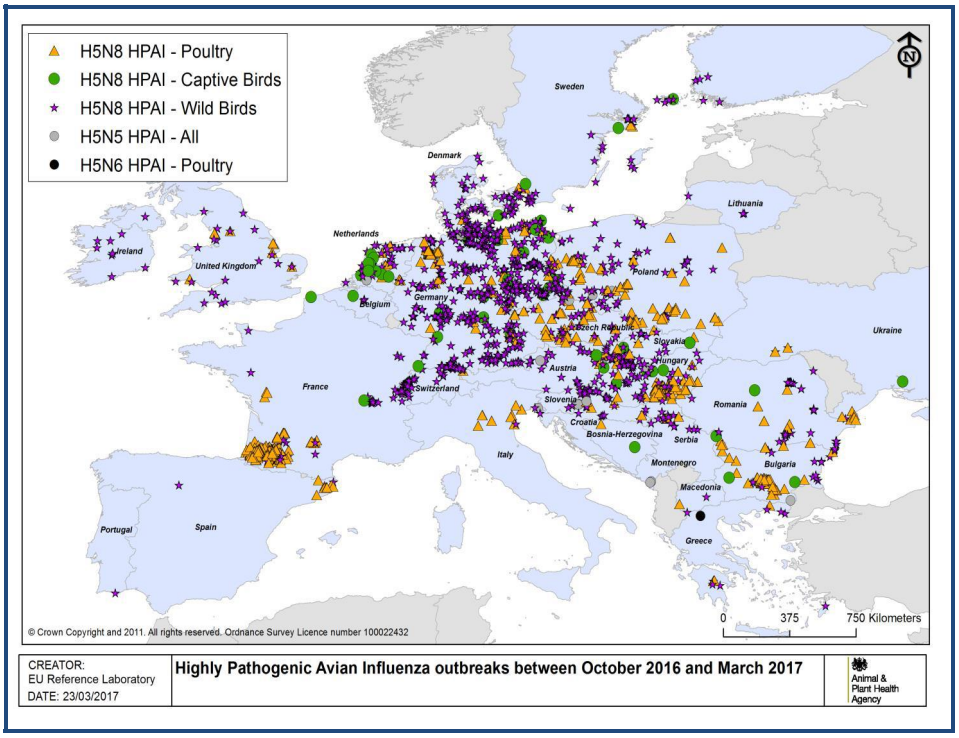

Figure 3: Highly pathogenic avian influenza outbreaks in Europe 2016-2017
prepared by EURL for Avian influenza, modified by source APHA-Weybridge, 2017

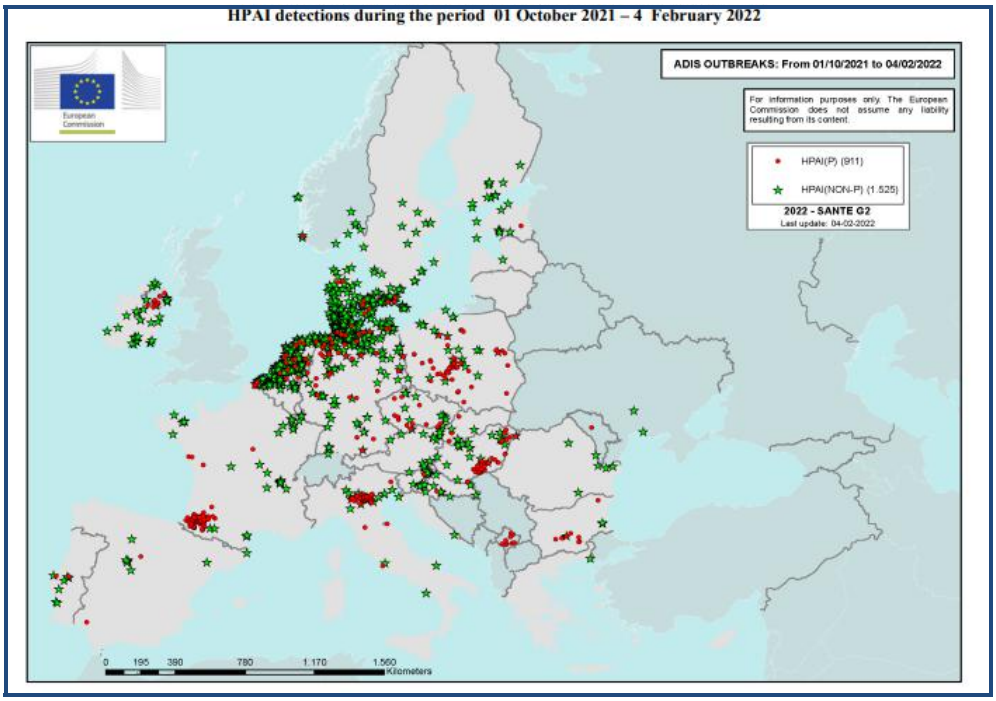

Figure 4: Highly pathogenic avian influenza outbreaks in Europe 2021-2022, modified by source Europa.eu, 2021

2. Aetiology and characteristics of the virus

Avian influenza viruses are type A orthomyxoviruses characterized by antigenically homologous nucleoprotein and matrix internal proteins. AIVs are so far divided into 16 hematogglutinin (H1-16) and 9 neuraminidase (N1-9) subtypes. Moreover, two new subtypes of HA and NA have been recognized H17N10 H18N11 that do not infect birds at all only bats (Tong et. al 2013) (Nathaniel et al.2020).

2.1 Avian Influenza Virus classification and Structure of the AI virus

Avian Influenza viruses are RNA viruses in the family *Orthomyxoviridae* which includes 5 genera: Influenza A, B, and C viruses, *Isavirus* and *Thogotovirus*, as proposed by the International Committee on Taxonomy of Viruses (ICTV). They are enveloped negative stranded RNA viruses distinguished based on antigenic differences in nucleocapsid (N) and matrix (M) proteins (Wright P., Webster R., 2001).

Avian influenza (bird) is caused by influenza A virus, that are the most widespread and of importance, infecting different avian and mammalian species. The other types B and C are mainly human pathogens, Isavirus is regarded as fish pathogens and the Thogoto viruses are tick-borne arboviruses infecting human and livestock (Kuno et. al. 2001).

The virion morphology varies from spherical particles (about 75-120 nm) to filamentous forms (several length over 300 nm) and mainly depends on two specific amino acids of

matrix 1 protein. AIV is a single-stranded, negative sense RNA virus, enveloped, pleomorphic and its genome comprises 8 segments of RNA encoding 11 identified polypeptides. The key structure proteins of the virus are hemagglutinin (HA), neuraminidase (NA) on the viral surface and the matrix2 (M2) which are included in its envelope lipid bilayer, both HA and NA are surface glycoproteins. HA is responsible for the attachment to the cell surface and binds to sialic acid residues in cell membrane glycoproteins, triggering viral fusion and entry of the cell. NA cleaves terminal sialic acid from glycoconjugates present on respiratory mucins, cells, and progeny virions. This is important for the release of the virus from the infected cells as a result the spread of the infection all over the respiratory tract (Journal Vaccines, 2021).

The M1 matrix protein (HA NA and M2) structure of the coat of lipid envelope encircles the virion core. The inner of the virion consists of the ribonucleoprotein (RNP) complex, comprising the RNA segments layered with the nucleoprotein (NP), non-structural protein 2 (NS2) and the RNA dependent RNA polymerase consists of 2 subunits of polymerase basic (PB1 PB2). Also, Polymerase acidic protein (PA) is included. The M2 protein plays the role of triggering viral uncoating and acts as an ion channel. HA is considered a trimmer because of its most abundant surface, whilst NA exists as a tetramer contributing to a spherical structure liberalizing the lipid membrane. A typical structure of avian influenza A virus and the virus components are shown in Figure 5 and Figure 6.

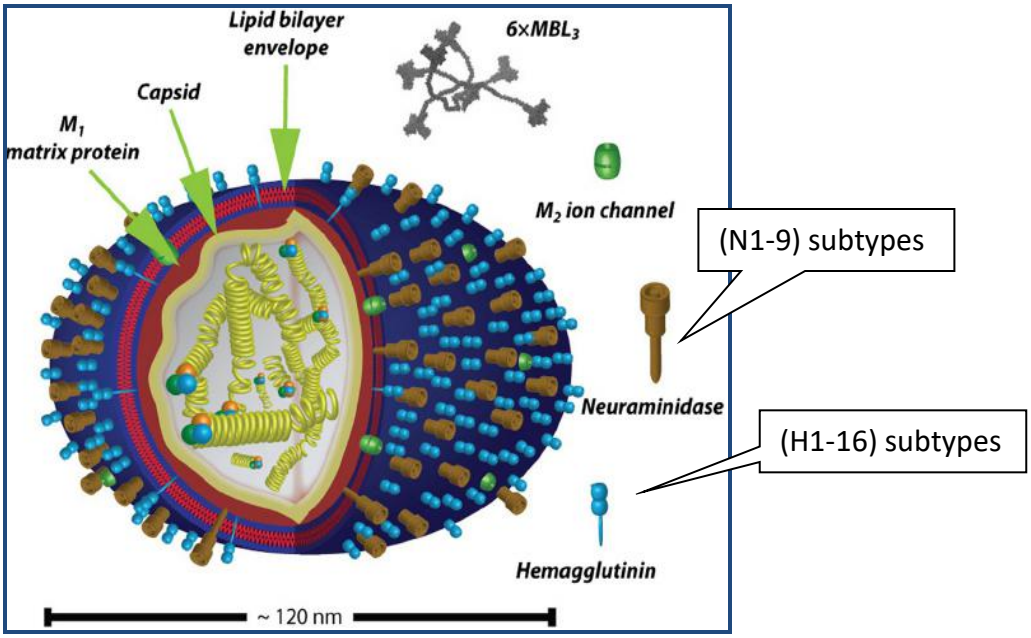

Figure 5: The structure of Avian Influenza Virus virion. Modified by source, Molecular and cellular therapies, 2013

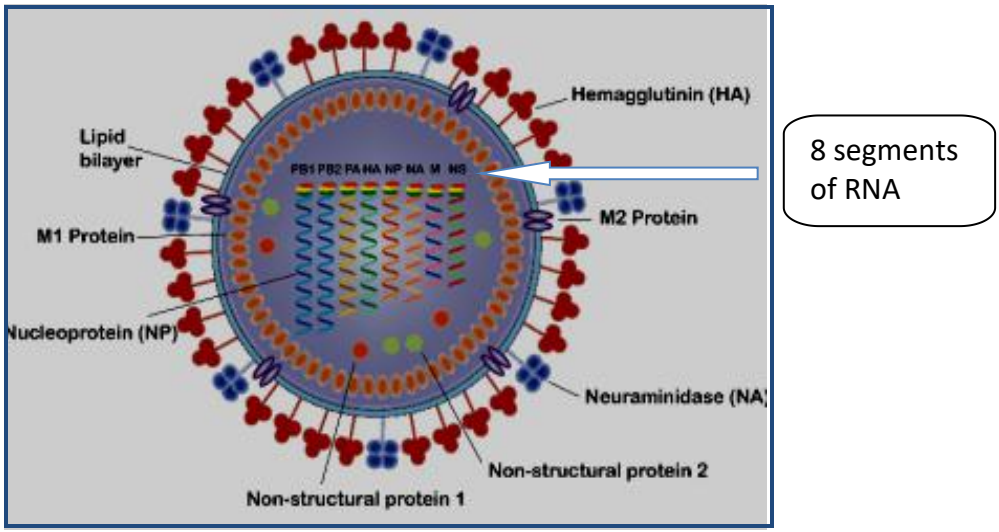

Figure 6: Schematic diagram of an influenza A virus, representing the virus components Modified by source, Journal Vaccines, 2021

2.2 RNA transcription and replication of the AI virus

After the virus enters into a cell, disassembly of the virus takes place, and the nucleus becomes the target site for transcription and replication of the viral genome. At this point the 8 segments of mRNA are synthesized in equimolar quantities. The amount of corresponding mRNA in the infected cells affects the amount of viral proteins synthesis.
The replication cycle of influenza A virus undergoes several stages (Figure 7). First the virus particle binds to the sialic acid cellular receptors which are present on the cell membrane surface, through its HA. The cellular receptors are connected to the carbohydrates of HA through glycosidic linkage, while 2 of them are very important for the specificity of HA. First the a (2,3) linkage which is abundant in the digestive tract in avian, in bronchial tissue in human, horses, monkeys and as well in the upper respiratory tract in swine, especially in the lung epithelium. The second is the a (2,6) linkage that is found in the upper respiratory tract in humans, as well in the trachea of bats and swine (M de Graaf, 2014) (Figure 8).
Then the virus enters susceptible cells by endocytosis and turns in endosome. Fusion and uncoating of viral particles are following, and release of the viral ribonucleoproteins (vRNPs) is followed into the cells cytoplasm, upon acidification of the endosome. The transcription of RNA into mRNA, which directs the viral protein synthesis, or in cRNA (complementary RNA) serving as template for the viral RNA genome synthesis, is mediated by a viral RNA polymerase complex active in the nucleus of host cells. The assembly and packaging of vRNAs into infectious virions involve several cellular compartments. Nuclear export is promoted by binding of M1 viral protein to RNPs, followed by the assembly of nucleocapsids in association with the cytoplasmic membrane and final new virion are released from the host cell surface (Figure 7) and spread to nearby cells.

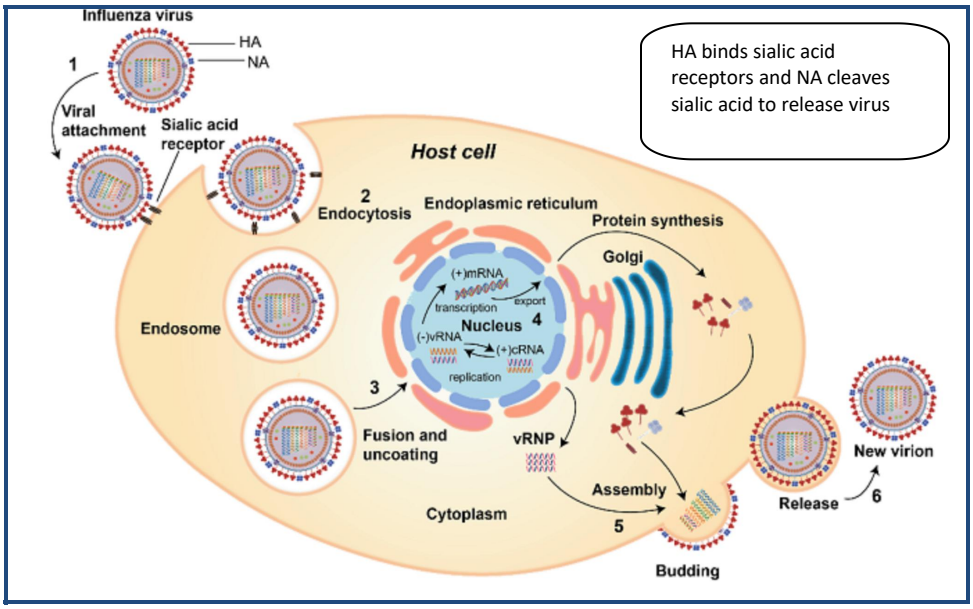

Figure 7: The life cycle of influenza A virus: Viral attachment, entry, fusion and uncoating, assembly and release of new virion**.** Transcription and replication of AIV. Modified by source: Journal Vaccines, 2021

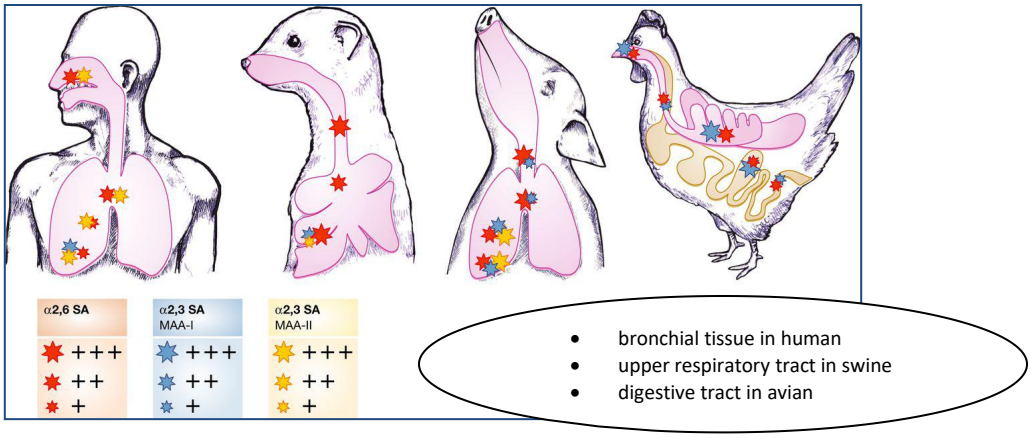

Figure 8: Sialic acid cellular receptors in human and animal. Modified by source: M de Graaf, 2014 EMBO Journal

2.3 Resistance of AI to physical and chemical factors

It has been established that AIV is inactivated by pasteurization and proper cooking (temperature of 70 ° C for 3.5 seconds) and in particular in eggs at 60° C for 188 seconds and in poultry meat for 507 seconds. The virus survives in frozen conditions and its resistance to substance such acids and bases, and it should be noted that AIV is inactivated by pH≤2. As for chemical factors, organic solvents, and detergents like sodium dodecyl-sulphate and deoxycholate inactivates AIV very efficiently. In presents of organics following substance are needed:

- Aldehydes (formaldehyde, glutaraldehyde)
- B-propiolactone
- Binary ethylene used after organic matter removal
- dilute acid pH<2
- Phenolics
- Oxidizing agents (potassium, sodium hypochlorite)
- Quaternary ammonium compounds (4%)
- Various lipid solvents

Surfaces that have been in contact with organic material might be cleaned with the aid of:

- Sodium carbonate (4 %) / sodium silicate (0.1 %)
- chlorine dioxide
- phenols (2%)
- Sodium hypochlorite (2-3 %)
- Sodium hydroxide (2 %)
- Strong oxidizing agents

(Committee of Foreign Animal Diseases, 2008)

Decontamination of the environment, the surfaces and equipment play a key role in the fight against the virus and of course the correct choice of proper disinfectants is a prerequisite. This is justified, while it has been proven that the virus survives in liquid faeces at temperatures of 25-32 ° C for 4 days, at temperatures of 20° C for 7 days and at 4° C for 30 to 35 days. In cases of poultry cages it has been determined that H7N2 LPAIVs persisted for more than 14 days in faeces. Moreover, in water conditions the virus showed his survival at 28 ° C for 26 to 30 days and at 17 ° C for 94 to 158 days. Additional, regarding composting the virus can be killed in 10 days in poultry carcasses (Joseph et al., 2016) (Office International des Epizooties, 2019).

3. CLINICAL FORMS OF AI AND POST-MORTEM FINDINGS

3.1 Clinical symptoms of AI in gallinaceous poultry

AIVs can affect a wide range of hosts including mammals (humans, pigs, horses, seals, whales, mink, dogs, felids, and civets), and therefore may pose a zoonotic risk. They can cause a variety of symptoms and lesions from subclinical infection to 100 % mortality, depending on the virulence of the AIV, the species affected and the subtype. The severity of clinical symptoms depends on the strain of the virus, the animal species and age, also the immunization status of the host, the co-infections with other infectious agents (*E.coli, Mycoplasma* spp*., Pasteurella* spp*., R. anatipestifer*) and the environment. It is also very important to assess the virulence of a given AIV pathotype (LPAIV or HPAIV) and its potential to cause disease in poultry or humans (Suarez, 2008).

According to the World organization For Animal Health (OIE), AI is defined as "*an infection of poultry caused by any influenza A virus with high pathogenicity (HPAI) and by H5 and H7 subtypes with low pathogenicity (H5/H7 LPAI)".* Furthermore, as the aquatic birds remain the main reservoirs of the virus, the virus cannot be completely eradicated, so observation and control of the pathogen in poultry and humans is necessary (Pello et. al, 2013). As for cagebirds, infections have been reported in passerine and psittacine even seldom. Although AIV mainly infects avian species, some strains are known to infect pigs, horses, dogs, cats, marine mammals, palm civets, stone martens and other species (Stallknecht, 1988) (Nathaniel et al.2020).

In domestic poultry the viruses are typically of low pathogenicity (LP) which is usually no cause clinical signs or causing subclinical infections, respiratory disease or even drop of egg production. A few AI are highly pathogenic (HP), causing severe systemic disease with organ failure and especially high mortality among poultry. Wild birds are natural hosts and play the role of reservoirs for all type of avian influenza viruses, becoming a major role in the evolution maintenance and spread of the viruses (see capital epidemiology).

The most subtypes of AIV (H1-H16) are low pathogenicity (LPAI), but some H5 and H7 AI viruses have high pathogenicity and are highly lethal for chicken, turkey and other gallinaceous domestic poultry. In wild birds AI causes mostly subclinical infections, expect for the H5 HPAI viruses of Eurasian lineage, which have been associated with mortality in wild and domestic waterfowl and species of wild birds generally.
The clinical symptoms the severity and mortality of the disease vary depending on the strain of AI and of course on the host species, the viral load and the infection route and also on the endemic status in the area. According to the World Health Organization and the World Organization for Animal Health, indentified cases are subject to notification for all highly pathogenic subtypes, as well as for H5 and H7 low pathogenic avian influenza subtypes.

3.2 Low pathogenicity Avian Influenza Viruses in poultry

High morbidity (more than 50 %) and low mortality (less than 5 %) are common in LPAIVs in poultry. LPAIVs typically produce respiratory symptoms like sneezing, coughing, nasal discharge, swollen infraorbital sinuses in poultry. Especially in ducks, turkeys and quail sinusitis is very common. The respiratory tract (trachea, lungs) has typically lesions of congestion and inflammation, and is usually accompanied by secondary pathogens like *E. coli* and *Pasteurella multocida*. Haemorrhages in the trachea, ovaries, salpingitis, enteritis, typhlitis and swollen kidney may also be noticed.

Breeders and layers show infertility or decreased egg production, ova rupture, involution, or mucosal oedema and exudates in the lumen of the oviduct. In some breeders and layer chicken renal failure and visceral urate deposition are occurring. Additionally, general clinical symptoms, such as fever, diarrhoea, lack of appetite and water consumption, huddling, ruffled feathers, and listlessness may be present. The mortality and morbidity are usually low in these cases unless secondary bacterial, viral infections or even environmental stressors accompanies. Nowadays, the H9N2 LPAIV is common in commercial and live bird market poultry in Asia, North Africa, and Middle East, but any subtype of LPAI viruses can occur sporadic.

3.3 High Pathogenicity Avian Influenza Viruses in poultry

HPAIVs cause systemic, severe disease with high mortality in chicken, turkey, and gallinaceous poultry, even of the absence of secondary infections. Indeed, the mortality rate range between 50 to 100 % and can be about 100 % in only few days. In peracute cases, the clinical signs or gross lesions are even lacking before death. However, in acute forms lesions include cyanosis, oedema of the head, wattle, comb, and snood (in turkey). Ischemic necrosis of wattles, comb or snood, red discoloration of the shanks and feet because of subcutaneous ecchymosis and oedema. Characteristic and common finding in necrotomy are the petechial haemorrhages on visceral organs and in muscles (Figures 9-10). Also, blood tinged oral and nasal discharges can occur. Very common is the greenish diarrhoea in severely affected birds. Moreover, fever, dehydration, lack of appetite and water intake, depression, decreased response to stimuli and rapidly progress of the infection during the first three days of infection (Kaleta, 2011).

Birds that may survive the peracute form of the disease (three to seven days) develop Central Nervous System (CNS) symptoms such as torticollis, paralysis, incoordination, opisthotonos and dropping wings. Of course, respiratory signs may also be seen like coughing, sneezing and rale. The microscopic lesions like oedema, haemorrhage, and necrosis in parenchymal cells in the organs, skin and CNS are highly variable. (Perkins, 2001) (Kaleta, et.al , 2005).

In the acute phase of the infection (between second and fifth day), swelling of the face, neck and legs, subcutaneous oedema of the feet and leg shanks, subcutaneous petechial haemorrhages of the non-feathered skin, ruffled feathers, cyanosis of the wattles and combs are some common lesions that are considered in clinical surveillance in order to

identify suspect HPAI cases. In some cases, hyperaemia and oedema of the trachea the eyelids and the conjunctiva are observed too (Kaleta, 2011).

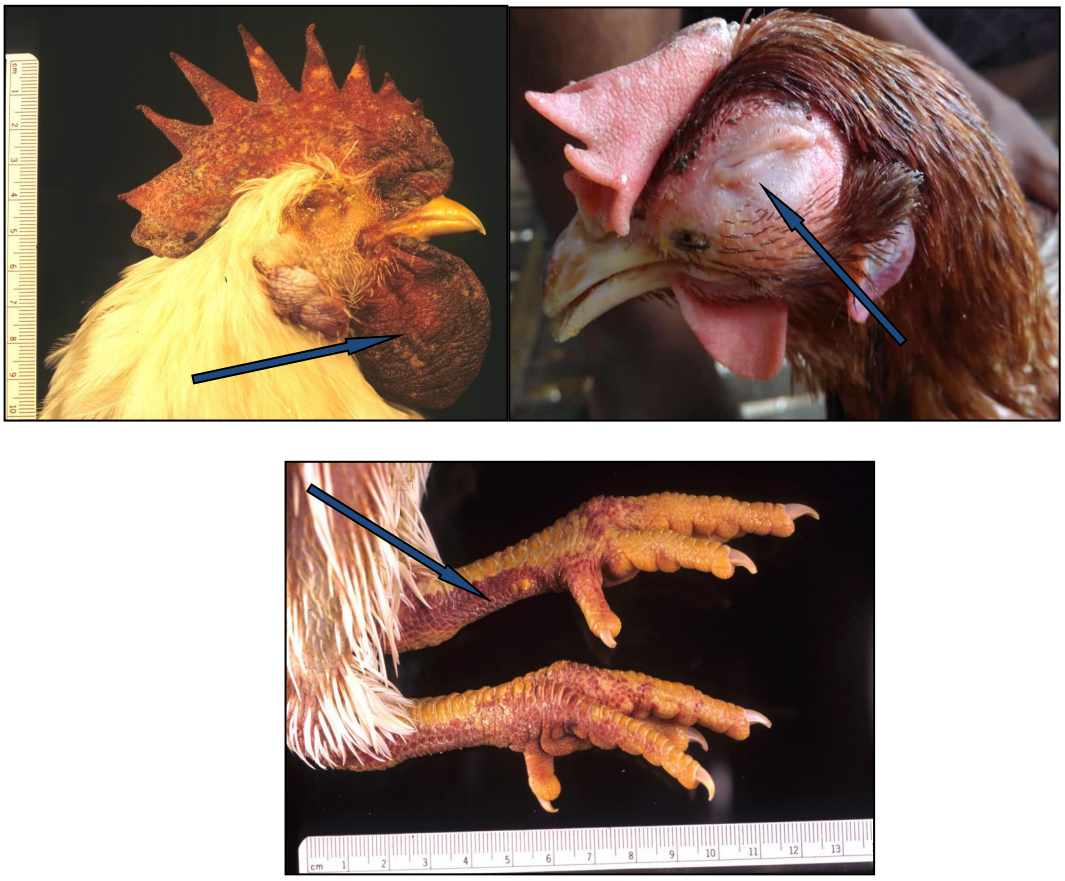

Figure 9: Hemorrhagic skin visible on the unfathered head regions and head edema of chicken with avian influenza. Hemorrhagic skin visible on the feet of a chicken with avian influenza. *Modified by source: Southeast Poultry Research Laboratory Dr. David E. Swayne.*

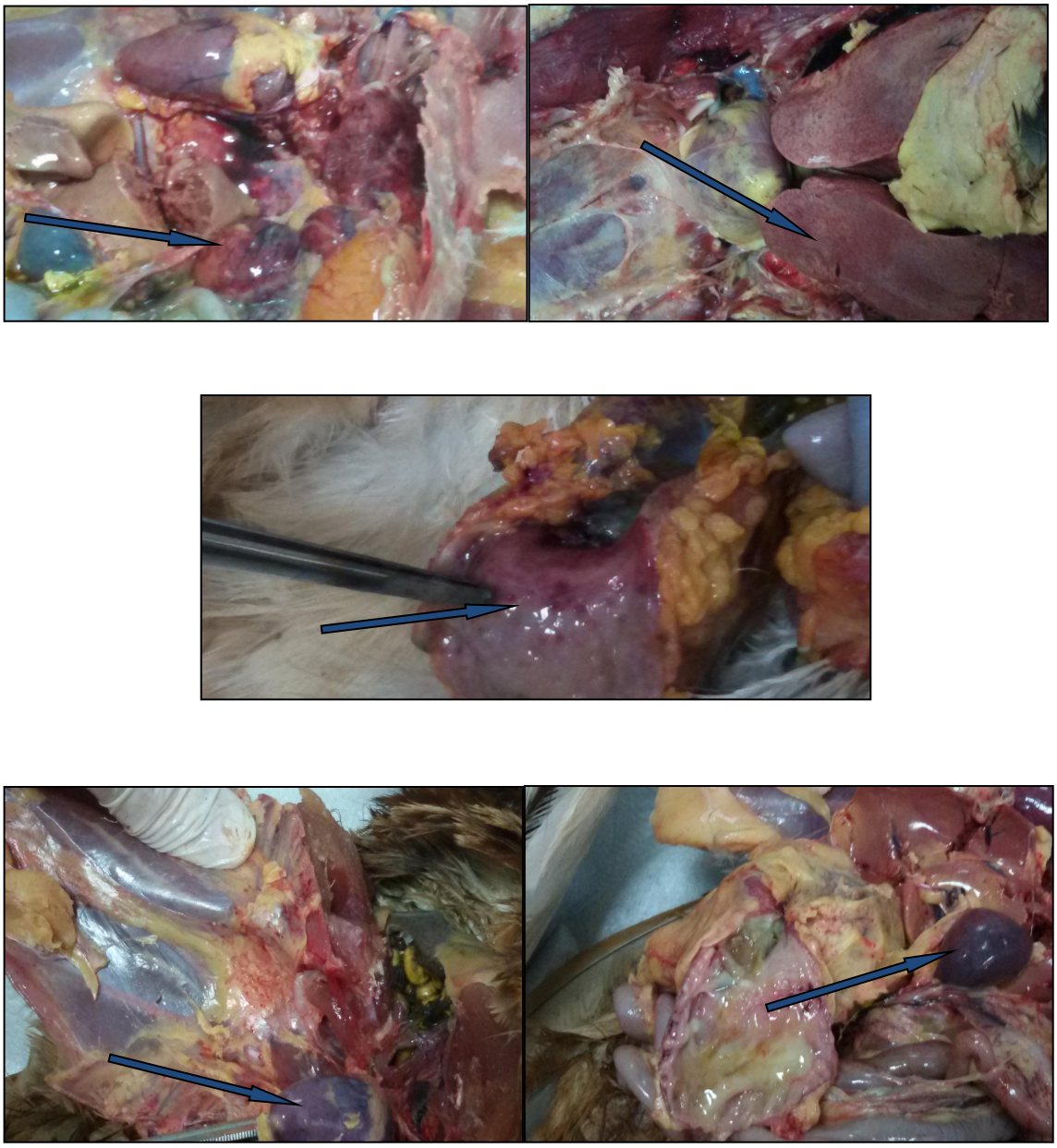

Figure 10: Chicken infected with AI: petechial haemorrhage in lever, oedema and haemorrhage on visceral organs of chicken infected with AI: Personal image archive

3.4 Clinical signs in wild birds, waterfowl, and ratites

In wild birds most infected show no obvious signs of the disease, but LPAIVs can affect the foraging of food and their migratory performance. Over time common AIV strains and their wild host population have developed an evolutionary balance, as a result non cause of disease or mortality. Periodically, wild ducks and geese have been identified as a source of virus introduction to poultry. Recombination or reassortment between LPAIVs in a common host does not necessarily lead to more virulence. HPAIVs in wild

birds show similar pathological changes like in poultry, which was described above (Figure 11).
HPAI infections in domestic ducks, leads to systemic infections and high virus titers in the respiratory tract and brain. Cardiovascular lesions, haemorrhage, diarrhoea, and multiple organ lesions are also noted (Figure 12). The mortality varies depending on the infection and the virus load of the disease. LPAI may cause asymptomatic infections mostly, or mild respiratory symptoms like sinusitis or conjunctivitis.

In Ratites decreased appetite, open mouth breeding, nervous system symptoms like torticollis, wing paralysis, tremor etc. have been noted. Also swollen throat and neck have been reported in ostriches (Chang et al., 2014). Haemorrhagic lesions of the intestine, peritonitis, enlarged pancreas and liver, oedema of the head and neck can accompany the pathological findings in HPAI. LPAIVs may cause respiratory symptoms and occasionally green diarrhoea in ostrich's emus and rheas (Alexander, 2000).

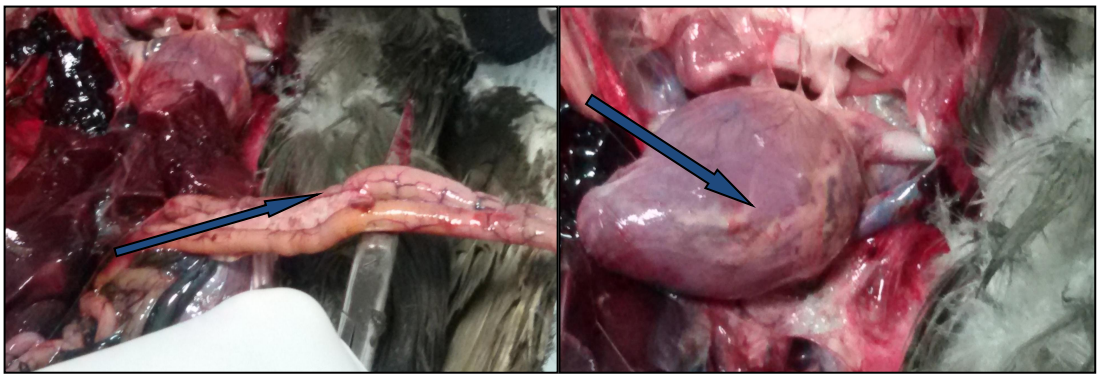

Figure 11: Haemorrhagic lesions of the intestine and cardiovascular lesions of duck infected with AI, Personal image archive

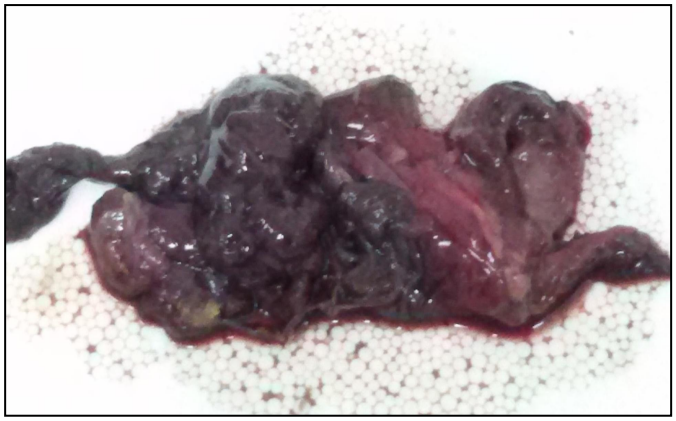

Figure 12: Totally haemorrhage of the intestinal track of swan infected with AI. Personal image archive

4. Diagnosis of Avian Influenza virus

The diagnosis is mainly based on serological and molecular test applied in biological samples from animals. Diagnosis requires blood serum samples, faeces or oropharyngeal and cloacal swabs, alternative fresh faeces obtained from live animals. In dead bird samples from trachea, lungs, air sacs, spleen, kidney, intestine, brain, liver, and heart should be collected and processed either separately or as a pool. Furthermore, faeces or oropharyngeal and cloacal swab are necessary (OIE, 2020).

A variety of diagnostic tests used for virus and antibody detection is described in Table 4. The selection of the appropriate test depends on different parameters, like the cost, the validity, the complexity, practicability, the speed of results, availability of materials and of course human resources.

Table 4: Test methods for the diagnosis of avian influenza virus and their purpose. Source modified: OIE Terrestrial Manual 2020.

Method of Diagnosis	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Virus isolation	√	√√√	√	√√√	√	-
Antigen detection	√	√	√	√	√	-
Molecular, Real-time PCR	√√	√√	√√	√√√	√√	-
Haemagglutination Inhibition	√√√	√√	√√√	√√	√√√	√√√
ELISA	√	√	√√	√	√√	√√
Agar Gel Immunodiffusion Assay	√	√	√√	√	√√	√√

√ may be used in some situations, but cost, reliability, or other factors limits is application
√√ suitable method
√√√ recommended method
- not appropriate for this purpose

4.1 Identification of the agent

4.1.1 Virus isolation

Virus isolation is the gold standard reference of AI diagnosis; its high sensitivity allows the isolation within 24 hours of the infection in individual birds and for several weeks post-exposure in flock cases. The virus isolation is not highly specific and additional tests are required for virus confirmation.

The preferred method of growing AIVs is the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative eggs (SAN). Eggs of 9-11 days incubation are used in this method, by obtaining the supernatant fluids of faeces or tissues suspensions and inoculated into the allantoic sac of the eggs. After that, the eggs are incubated at 37 ^{o} C for 2-7 days. The allantoic fluid from dead embryos is tested with a screening test like hemagglutination assay, a specific influenza A type test like AGID, and a molecular test like RT-PCR (for Matrix, H5 and H7) to confirm the presence of influenza virus. The fluids that test negative should be passaged in one further batch of eggs.

4.1.2 Assessment of pathogenicity

Assessment of pathogenicity is related with the term HPAI in chicken and denotes the intricacy of highly pathogenic strains. It describes the disease in chicken that develop all the typical clinical symptoms and are accompanied with high and rapidly mortality in the flock. Nowadays the HPAIV belong to the H5 and H7 subtypes, and studies shown that H5/H7 LPAIVs are potentially pathogenic. The criteria set up by the OIE, determining the pathogenicity are the following:

a. Any AIV that is lethal for six, seven or eight out of eight 4- to 8-week-old susceptible chicken within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria free, infected allantoic fluid
b. or any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2

The OIE has set up a classification system to identify influenza A virus and for which disease reporting and control measures should be taken:

a. All isolates from poultry that meet the above criteria are designated as HPAIV and are notifiable
b. H5 and H7 isolates from poultry that are not highly pathogenic for chicken and don't have a HAO cleavage site amino acid sequence similar to any of those that have been observed in HPAI viruses are designated LPAI H5/H7 and are notifiable
c. HPAIV and LPAIV H5/H7 in poultry are termed ''avian influenza" and are notifiable. But non H5/ H7 influenza are not ''avian influenza" and not notifiable (H1-4, H6, H8-16), fort the purpose of the *Terrestrial Code.*
d. High pathogenicity of influenza A viruses in other birds than poultry, including wild birds are notifiable

4.2 Antigen capture and molecular techniques

Method of choice at least for the initial diagnosis of influenza remains the conventional virus isolation and characterization. However, conventionally methods are considered slow, costly, and labour intensive. Enormous development has been done in other molecular and diagnostic techniques that are applied for the diagnosis of influenza infections.

4.2.1 Antigen detection

Several commercially AC-ELISA kits are available to detect the presence of Influenza A viruses in poultry. Most of them are based on immunochromatography or enzyme immunoassays, using a monoclonal antibody against the nucleoprotein, able to detect any influenza a virus. The biggest advantage of the method is that the demonstration is done in 15 minutes. The disadvantages are the low sensitivity, they are not validated for different species of birds and the subtype identification is not possible. Moreover, the method is expensive and should only be interpreted on a flock basis and not as an individual bird basis. In addition, because of the low sensitivity, antigen detection is used for field screening of high mortality clinical cases for influenza A followed by confirmation using a more sensitive laboratory method like RT-PCR.

4.2.2 Direct RNA detection

For some time now, molecular techniques have been developed to the detection and characterization of influenza A viruses from clinical specimens of infected birds. It is mandatory by using sensitive molecular detection methods that allow rapid direct detection of viral RNA for diagnosis and confirmation of infections with viruses, and stringent protocols are in place preventing the cross contamination between samples. Moreover, RNA detection tests should also be validated to the OIE standard using clinical material fit to purpose for application in field diagnostic settings, which include the use of internal standard for example.

The preferred molecular detection for influenza virus is the real-time RT-PCR. This approach is adopted in the national reference laboratory of avian influenza virus in Europe. It is based on initial generic detection of influenza A virus in the clinical specimens, primarily targeting at first the matrix M gene, being highly conserved for all influenza A viruses. The specific RT-PCR for testing H5 and H7 subtypes is followed. The RT-PCR usually is based around the hydrolysis probe or TaqMan (Nagy, Slomka, 2020) method for generation of the target specific fluorescence signal, offering a rapid method with specificity and sensitivity comparable to virus isolation. For influenza outbreaks management where the time to make decisions is crucial for the veterinary authorities this method is ideal. These RT-RCR systems operate in a 96 well format and are combined with high throughput robotic RNA extraction from specimens (Aquero et al. 2007). Validated protocols for the simultaneous detection and typing of H5, H7 and H9 have been developed, to be able the investigation many types of HPAI clinical specimens from different laboratories from Europe, Asia, and Africa since 2005 (Slomka et al., 2007, Monne et al., 2008). Additionally, currently the preferred method for the surveillance

programs is RT-PCR, whereas provides rapid and sensitive diagnostics for influenza A virus H5 and H7.

4.3 Serological tests

4.3.1 Enzyme-linked immunoassay (ELISA)

ELISA is designed to measure the relative level of antibody to AIV in avian serum. The assay is performed on 96- well plates that have been coated with AIV. Thanks to the variation and severity of clinical symptoms, serological testing produces significant advantages to the detection of infected birds. The commercial kits of ELISA, which ought to be certified by the OIE, have an indirect or competitive/blocking format, are validated and accustomed detect specific antibodies, by allowing these antibodies to compete for antigen binding sites with a monoclonal antibody against an epitope on the nucleoprotein that's conserved in all influenza A viruses. Monitoring the status of infection in flocks to AI is connected by the measurement of antibody to AIV in serum. Disadvantages of the method are the low of sensitivity, not validation for various species of birds and subtype identification is also not possible. ELISA may be a moderate cost method and is amenable to high through put screening for influenza A virus infections. Moreover, samples that are tested positive must be still tested by HI test for subtypes H5 and H7.

4.3.2 Agar Gel Immunodiffusion Assay (AGID)

AGID visualizes the immunoprecipitation reaction of the AIV antigen after diffusion in an agar matrix. For these purpose, concentrated virus preparations, containing both matrix and nucleocapsid antigens are used. The matrix antigen diffuses more rapidly than the nucleocapsid antigen. The tests are widely used for the detection of specific antibodies in chicken and turkeys' flocks as an indication of infections. However, in other bird species these tests are less reliable. This assay may be a low-cost screening test for detection of generic influenza A infections, but still HI test for subtyping to H5 and H7 influenza virus is followed.

4.3.3 Hemagglutination inhibition tests

The hemagglutination inhibition (HI) test, is a classical laboratory procedure, shown high sensitivity for detecting subtype specific antibodies to the HA antigen in poultry. It comprises a quantitative method, applied frequently for the evaluation of antigenic relationships between different AI virus isolates of the same subtype. The assay is relatively inexpensive, utilizing standard labour equipment; less complicated than molecular tests and is easily completed in several hours. Moreover, extensive laboratory support for the production and optimization of reagents is required.

4.4 Differential Diagnosis

The way in which AI in birds presents itself clinically varies from case to case, depending on different parameters as already mentioned in the presentation of the clinical stages of the disease. The diagnosis only on clinical symptoms is problematic since the symptoms may be similar in other poultry diseases, so the rule is that no diagnosis is definite before laboratory confirmation. The differential diagnosis of AI in birds includes for LPAIVs the following diseases:

- Infectious bronchitis (IBV), infectious laryngotracheitis, low virulence Newcastle disease, infections by other paramyxoviruses (acute to subacute viral diseases), causing respiratory or enteric signs
- Mycoplasmosis, infectious coryza, ornithobacteriosis, turkey coryza and the respiratory form of fowl cholera (bacterial diseases), causing respiratory signs usually coughing, swelling of the sinuses.
- Fungal diseases like aspergillosis causing respiratory signs

For the HPAI (high mortality) the differential diagnosis includes following diseases:

- virulent Newcastle disease (NDV) causing sudden high mortality or haemostasis in wattles and combs
- peracute septicaemic form of fowl cholera causing swelling of the combs and wattles
- heat exhaustion causing sudden high mortality
- severe water deprivation causing sudden high mortality
- poisoning causing sudden high mortality

Laboratory confirmation is required to make an accurate diagnosis, choosing the appropriate method out of those already described.

5. Control - Eradication and Prevention of AIV

5.1 Control measures and Legal framework in Europe

The best way to control the entrance of the virus in a county is the implementation of special measures. No EU member country wants to deal with viruses that cause enormous socioeconomically affects. For that reason, the EU has adopted a common policy to control AI, through a surveillance program and has lay down precautionary measures to prevent and measures to control and eradicate the disease in case of appearance. These measures are applied in domestic, systemic, backyard poultry also in wild birds.

Since 2003, all EU member states must implement passive surveillance programmes for the AI, aiming to detect infections with low pathogenic avian influenza viruses of H5 and H7 subtypes in poultry which have the potential to mutate to highly pathogenic form of

the virus. The spread of the HPAI H5N1 of the Asian lineage has triggered enhanced surveillance and early detection systems in poultry and in wild birds. The surveillance for avian influenza is compulsory, and in accordance with implementing Regulation EU 2020/690, highly pathogenic and infection with low pathogenic AI viruses are subject to Union surveillance programmes, which are relevant for the union. Member States are obligated to report the results of the surveillance to the Commission, in accordance with implementing Regulation EU 2020/2002. The data are submitted electronically via the Animal Disease Information System (ADIS). Furthermore, surveillance in poultry and wild birds must be implemented on the entire territory of all EU member states and in accordance with the provisions laid down in Annex II to Delegated regulation EU 2020/689 (ec.europa.eu)

The main key prevention measures are described in the Council Directive 2005/94/EC, laying down the surveillance program that is compulsory, the measures that have to be followed for the control of AI accompanied by the Commission Decision 2010/367/EC were guidelines on the implementation of surveillance programmes AI have the objective to timely detect HPAIVs. In this framework the measures must be taken serious by each member state for the evolution of AIV in Europe. Furthermore, the diagnostic procedures that are followed for AIV are laid down in the Diagnostic Manual for Avian influenza 2006/437/EC, as provided for in Council Directive 2005/94/EC, which sets out guidelines to carry out the collection and transport of samples, laboratory protocols and criteria for evaluation of the results of laboratory tests for an efficiency diagnosis of AIV.

The Council Directive 2005 which establishes the minimum measures to be applied within EU for the control of AIV, includes 69 Articles in total, and at followed should be kept as more special on mind for the HPAI viruses:

- ✧ Article 5 Avian influenza Notification
- ✧ Article 6 Epidemiological inquiry
- ✧ Article 7 Measures to be applied in cases where AI on a holding is suspected
- ✧ Article 11 Measures to be applied in cases where AI on a holding is confirmed
- ✧ Article 14 Measures to be applied in cases where AI on a holding is confirmed in various production units
- ✧ Article 15 Measures in contact holdings
- ✧ Article 17 Measures in protection and surveillance zones
- ✧ Article 22 Prohibition on the movement and transport of birds, eggs, poultry meat and carcases
- ✧ Article 28 Cleaning and disinfection
- ✧ Article 34 Biosecurity measures
- ✧ Article 36 Measures to be applied in slaughterhouses

In general, the Directive describes the measures in case of infected farms, the infected area and the provision to apply on the holdings in that area. The control and eradication measures include Establishment of protection zones (in a radius at least of 10 kilometres around the holdings), and a surveillance zone (in a radius of at least 3 kilometres around the holding) enhanced epidemiological surveillance measures and investigation, tracing of poultry and the most important for the eradication is the so called stamping out in

infected holdings. These seems to be until now the most effective form of eradication and the countries can declare as free in a short relative time of the virus. These measures are applied in combination with strict quarantine to contain the disease, biosecurity measures on farms holdings and of course the movement of poultry products is controlled too. The mentioned measures should be accompanied by awareness of the public using campaigns, training and information about the risks related to the virus for the stakeholders plays an important role also.

The new Commission Delegated Regulation EU 2020/687, which relates to rules for prevention and control of certain listed diseases and the Regulation EU 2016/429 also known as the "Animal Health Law", sets out different way of handling AI cases, especially with regards to the restocking in surveillance zones. The latter is now more difficult and should be revised to offer more flexibility. On the one hand, high biosecurity standards and strong surveillance system are the only way to protect the flocks. On the other hand, a strong discussion on the potential use of vaccination against HPAI is ongoing. The EU authorities EFSA (European Food safety Authority) and the ECDC (European Centre for Disease Prevention and Control) that are responsible for animal and human health work closely with the World organisation for Animal Health (OIE), the World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) in the fight against avian influenza for the next epidemic waves using a One Health approach.

5.2 Prevention of the disease

Avian influenza spreads through direct contact with infected birds, contaminated feed, water, equipment and even clothing. Therefore, biosecurity is the first and most important means of prevention at least at farm level, and poultry producers are advised to take special measures to prevent the introduction of the virus in their flock.
That is why the EU has developed a prevention strategy which is adapted by each member state. In specific the poultry farms systems are distinguished in domestic systemic and backyard poultry.

Biosecurity is defined as all management and physical measures aimed at reducing the risk of introduction, development and spread of pathogens from, to and within an animal population or a farm, area, means of transport or any other establishment and location. Especially in the case of avian influenza, systematic observance of biosecurity measures is vital, given the speed of transmission of the virus, the peculiarities in its epidemiology and the magnitude of the economic impact that it's possible uncontrolled spread brings about. In Greece, the following measures are established:

<u>In domestic poultry farms, biosafety- biosecurity measures include:</u>

- Housing of all poultry in closed, fenced areas, covering the openings of poultry houses with screens, nets or other appropriate protective covers, which will exclude contact between domestic poultry and wild birds' attractants

- The separation of the rearing of goose and ducks from other poultry
- Good cleaning of the surrounding area of the farm, so that there are no feed and water that attract any infected birds, making sure all water and feed to poultry is in an enclosed space inaccessible to any infected wild bird
- Minimise direct and indirect contact between individuals in rearing and between poultry and captive birds in the event of suspicion of the disease
- The non-discharge of dead birds into environment
- Cleaning and antisepsis of the hands of people engaged in the care of the poultry
- Cleaning and disinfecting shoes in containers with common disinfectants (which are regularly renewed) before entering and leaving the hen house

In systemic poultry farms, biosafety- biosecurity measures include:

- House of all poultry in closed establishment and fenced areas according to the type of breeding
- The fencing of the poultry facilities to ensure that the entrance to them will be done from a controlled point and after mandatory disinfection of the wheels of incoming vehicles. For this purpose, at the entrances to the farms there must be trenches with a disinfectant solution renewed every 24 hours
- Minimizing the number of vehicles entering the farm as strictly necessary and providing documentation on the prior cleaning and disinfection of such vehicles
- The retention of the vehicles of staff, visitors, etc., outside the fencing of the holding
- The planned, regular cleaning and disinfection of the vehicles on the holding, in addition after transport
- The disinfection of the wards and all equipment immediately after the end of each breeding, with special attention to the removal of all organic matter (use of appropriate cleaners and water under high pressure)
- The restriction of access by wild birds in the buildings and places where poultry live, and feed and eggs are stored, using the necessary means (sings, nets, etc.)
- The implementation of systemic programmes of effective vermin control
- The repopulation of individual or all the wards on the holding with poultry from flocks or holdings free from influenza virus and other pathogenic microorganisms
- Avoiding the use of water from surface water, or watercolours. Failing that, subjecting the water to appropriate treatment and regular disinfection to inactivate pathogenic microorganisms, in particular influenza and Newcastle disease viruses
- The separation of a clean and unclean zone in the poultry farm and the maintenance of the changing rooms in the space between them, so that people enter the poultry housing area from there
- The carrying out of complete and detailed records of mortality, disease diagnosis, treatments, and vaccinations for each flock, which are always available to the Veterinary Authorities
- The immediate removal of sick and dead birds from the wards or shelters of poultry, as well as their adequate and effective disposal
- The immediate adoption of eradication measures in the event of a confirmed case in the farm, in accordance with the instructions of the local veterinary authority

- The avoidance of successive visits to different poultry farms with a <72-hour interval, which is particularly recommended for staff collecting and loading poultry for slaughter
- Limiting the number of visits to the farm to what is strictly necessary and recording them in detail
- The establishment of a protocol of personal hygiene and disinfection for the staff and visitors of the farm at each entrance, during the change of activity, before the start of work, after breaks and leaving holding
- Cleaning and hand disinfection before entering the poultry farm, wards, and other premises, as well as before leaving the farm
- In the case of visitors, use each time of clean clothing and footwear recommended, belonging to the holding (e.g., disposable) and to remain on it after the end of the visit
- The mandatory passage through disinfectant foot baths before entering the farm, chambers, and any other area, with regular renewal of the disinfectant
- The limitation to a minimum of time of contact between individuals in rearing and birds in the event of suspicion of the disease
- The correct and effective carrying out of the necessary disinfections. Necessary conditions are the use of appropriate disinfectants witch viricidal action (approved by the national medicines agency), the prior adequate cleaning of each surface, as well as the sufficient duration of application of the disinfectant to ensure its action

Other biosafety-biosecurity measures:

- It is forbidden to use birds of the classes Anseriformes and Charadriiformes, as baits during the hunting of wild birds
- It is forbidden to gather poultry and other birds in open air exhibitions, markets, shows and cultural events, as well as to organise their exhibitions and competitions in open air and open spaces
- It is forbidden to sell poultry and other birds of any age and any kind in open air farmers markets. The disposal of poultry and birds to houses is allowed after a short stop of the vehicle for their transport and for a quick time for their unloading
- Establishments of marketing poultry for domestic livestock must be approved by the veterinary authorities and should comply with the biosecurity rules, while it is forbidden to keep poultry out of the establishment
- The rearing, handling and disposal of farmed poultry for marketing as eco-cements must be carried out in accordance with specific veterinary terms and conditions
- Zoo parks or similar establishments keeping captive birds must take all necessary measures to avoid disease transmission to them (permanent keeping of birds in cages all the openings and roofs of which will prevent contact with wild birds by using appropriate protective metal or material of constructed nets with a small opening, or other means that achieve this goal)

Hunters who encounter wild birds during the hunting season, potentially infected with avian influenza virus, should:

- Strictly follow personal hygiene measures when cleaning, preparing, and cooking feathered game

- Clean and disinfect their vehicles and hunting equipment (clothes, footwear, etc.) to prevent the spread of avian influenza viruses from wild birds to poultry
- Not feed poultry or pets (dogs, cats) with raw feathered game or their residues
- Not throw away in the environment corpses or remains of wild birds

Measures that are taken in case of the present of the disease:

- If the presence of avian influenza in domestic or systematic poultry farms is confirmed, all birds must be killed immediately. In addition, their products feed, bedding and all materials and waste that may have been contaminated are destroyed, and in the end cleaning and disinfection operations are carried out.
- Furthermore, in the case of contaminated farm restrictions are imposed on the movement of poultry and their products in the farm as well as in all farms within a given zone by the veterinary authorities.

5.3 Avian Influenza (bird flu) Vaccination

Vaccination against avian influenza is restricted by legislation in Europe and it is not a routine control measure of protecting against and controlling an avian influenza outbreak. Usually, preventive vaccination is widely used to control other diseases in avian, but for these disease disadvantages have been related to currently available avian influenza vaccines (ecdc.europa.eu, 2022).
The key Disadvantages are:

- ✧ vaccinated birds can transmit avian influenza during an infection with the virus
- ✧ must match the vaccine and field hemagglutinin subtype to get protection
- ✧ influenza viruses mutate very rapidly, making the vaccine less useful
- ✧ practical limitations (individually injecting each bird, 3 doses are required)
- ✧ no proven efficacy of the vaccines in species such ducks, geese, game birds
- ✧ differentiation of infected and vaccinated birds is difficult
- ✧ welfare implications for birds are in place (increased handling in speed conditions)
- ✧ could lead to false sense of security because of relaxation of biosecurity and vigilance
- ✧ risk for workers from handling of birds

6. Epidemiology - Outbreaks in Greece

The first cases of HPAI virus H5N1 in Greece, was reported in January 2006. The HPAI outbreaks occurred in six different regions of the country (Central Macedonia, Eastern Macedonia and Thrace, Western Macedonia, Central Greece, Peloponnese, and Aegean

Islands). Totally 16 swans (*Cycnus olor*) and one red- breasted wild goose (*Branta ruficollis*) were found dead on coast sides in Thessaloniki, Pieria, Chalkidiki, Rhodopi, Pella, and the Evia region unit. The first introduction of the virus could not be determined, but it is suspected that the infection might have originated from a group of swans arrived twenty days ahead probably from the north, due bad weather conditions (OIE 2017). The veterinary authorities introduced and applied all the measures imposed by the European Commission Decision 2006/115/EU immediately after the positive samples' notification. During that period no human cases were reported according to the WHO.

No positive findings for AI were confirmed either in poultry holdings or in wild birds until December 2016, where another mute swan (*Cycnus olor*) was found dead in the Evros river delta in the regional units of Eastern Macedonia and Thrace. A new HPAIV strain was identified namely H5N8, which was the first case of dead HPAIV H5N8 in wild birds species (OIE 2017). Reports on wild birds found infected first concentrated at Evros, then in Florina, Arkadia, island Rhodes and Rhodopi regional units. The epidemic curve showed two peaks. The first occurred in early January and the second in February. Finally, 9 cases of HPAIV infections in wild birds were recorded, most of them caused by subtype H5N8, while one was type H5N5 and all investigated birds were HPAIV positive. The distribution of HPAIV in combination with the affected bird's species is presented in Figure 13, Table 5 and 6.

Table 5: Poultry holdings outbreaks 12 January 2017 -23 March 2017 in Greece

LOCATION (REGIONAL UNIT)	CONFIRMATION DATE	PATHOGENICITY & SUBTYPE	PRODUCTION TYPE	POULTRY SPECIES
ARKADIA	12/01/2017	HPAI H5N8	COMMERCIAL	LAYING HENS
ARKADIA	25/01/2017	HPAI H5N8	BACKYARD	DUCKS AND CHICKENS
RODOPI	26/01/2017	HPAI H5N8	BACKYARD	DUCKS AND CHICKENS
FLORINA	16/02/2017	HPAI H5N6	BACKYARD	CHICKENS
ARKADIA	16/02/2017	HPAI H5N8	BACKYARD	GEESE
KOZANI	23/03/2017	HPAI H5N8	BACKYARD	CHICKENS ANDTURKEYS

Table 6: Wild bird outbreaks 2016 - 2017 in Greece

LOCATION (REGIONAL UNIT)	CONFIRMATION DATE	PATHOGENICITY & SUBTYPE	WILD BIRD NUMBER	WILD BIRD SPECIES
EVROS	19/12/2016	HPAI H5N8	1	*Cygnus olor*
EVROS	26/01/2017	HPAI H5N5	1	*Cygnus olor*
FLORINA	27/01/2017	HPAI H5N8	1	*Anseranser*
ARKADIA	27/01/2017	HPAI H5N8	1	*Cygnus olor*
ARKADIA	02/02/2017	HPAI H5N8	1	Pica pica
DODEKANISA (RHODES)	06/02/2017	HPAI H5N8	1	*Cygnus olor*
EVROS	16/02/2017	HPAI H5N8	1	*Cygnus olor*
RODOPI	16/02/2017	HPAI H5N8	3	*Cygnus olor*
ARKADIA	16/02/2017	HPAI H5N8	2	*Cygnus olor*

In addition, outbreaks in captive birds were notified (1 case of HPAI H5N8 in commercial poultry holding and 4 cases of HPAI H5N8 and H5N6 in backyard farms). After the decline of the epidemic in spring 2017, no other cases in wild birds were detected in 2017.

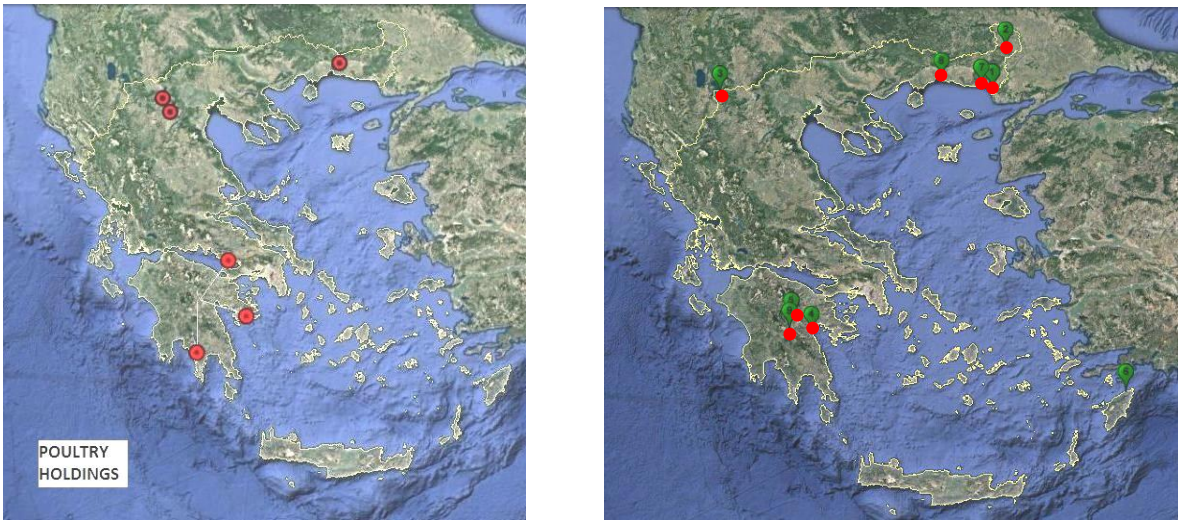

Figure 13: Poultry holdings outbreaks on the left and Wild Birds outbreaks on the right 2016-2017 in Greece

In Greece in 2021, outbreaks of virus H5N8 subtype were confirmed in wild birds, i.e. pelicans (*Pelecanus crispus*) in Serres, Florina and Kastoria regional unit and muted swans (*Cygnus olor*) in the Evros river delta in the regional unit of Eastern Macedonia and Thrace. All birds were found dead. In total 5 cases of HPAIV infections in wild birds were recorded, most of them caused by subtype H5N8, while one was type H5N1 and all investigated birds were HPAIV positive. The distribution of HPAIV in combination with the affected birds species is presented in Figure 14 and Table 5. In addition, no outbreaks in captive birds were notified and the veterinary authorities tried to focus on checking the biosecurity measures and on implementing the AI surveillance program in wild birds and in poultry.

On February 2022, outbreaks of H5N1 subtype were comfirmed in wild pelicans (*Pelecanus crispus*) in Florina and (*Cygnus olor*) in Kastoria regional unit. All birds were found dead and especially in the protected area of Lesser Lake Prespa a total of 1003 dead Dalmatian pelicans had been recorded in the Lesser Prespa colonies on March. Also outbreaks of H5N1 were confirned on March in wild pelicans in Kilkis regional unit in the area of Doirani Lake. In total 16 cases of HPAIV infections were confirmed by the National Reference Laboratory for avian influenza viruses, all caused by H5N1. The distribution of HPAIV in combination with the affected birds species is presented in Table 7.

The veterinary authorities immediately started implementing biosecurity measures fort the poultry sector and to minimize the spread of the disease to other wetlands, also to protect the next wave of pelicans and others migratory waterbirds and raptors arriving soon. The effective monitoring and clearing of the area of remaining carcasses is challenging concidering the high disturbance of the still unaffected colonies and the low temperatures by continious snow fall in the area for unusually severe weather conditions for this time of the year. It has to be noticed that the lake Prespa breeding colony has crown to be one of the most important for the global population of the dalmatian pelicans.

Table 7: Wild bird outbreaks in 2021 - March 2022 in Greece

LOCATION (REGIONAL UNIT)	CONFIRMATION DATE	PATHOGENICITY & SUBTYPE	WILD BIRD NUMBER	WILD BIRD SPECIES
SERRES	01/04/2021	HPAI H5N8	3	*Pelecanus crispus*
FLORINA	08/04/2021	HPAI H5N8	1	*Pelecanus crispus*
KASTORIA	08/04/2021	HPAI H5N8	1	*Pelecanus crispus*
KASTORIA	20/04/2021	HPAI H5N8	2	*Pelecanus crispus*

EVROS	10/12/2021	HPAI H5N1	1	*Cycnus olor*
FLORINA	05/03/2022	HPAI H5N1	4	*Pelecanus crispus*
KASTORIA	05/03/2022	HPAI H5N1	4	*Cycnus olor*
KASTORIA	10/03/2022	HPAI H5N1	4	*Pelecanus crispus*
KILKIS	30/03/2022	HPAI H5N1	4	*Pelecanus crispus*

Figure 14: Wild birds outbreaks on April 2021 in Greece

7. Conclusions

The avian influenza virus continues to demand the attention of the international community, being a crisis of global importance, since the virus continues to circulate around the world, showing high viral genetic diversity and will not probably be eradicated soon. The role of wildlife, domestic ducks, and pigs in the transmission among animals, still not fully understood after so many years, make it more urgent to conduct more research, to understand the ecology and trends in virus diversity and prevalence. Furthermore, the threat to human and animal health continues to exist, even much progress has been made in early detection and reaction, measures and well-structured, long-term surveillance should remain very strength in all countries.

However, in infected animals the best way of controlling the disease is the stamping out method, recommended by FAO and OIE. Any vaccination strategy should be made by

each country based on its own situation, considering the ability to detect and to react to disease as early as possible using transparent timely notification, meaning a good institutional framework and sound legislation that supports the veterinary services in the countries. Moreover, any vaccination strategy should be developed consider the opinion of stakeholders and the private sector of poultry and their production. In addition, vaccination should be carried out under the supervision of official veterinary services and very important in parallel with surveillance strategy, to be able to identify and monitor the circulating virus, but also the response to vaccination.

The collaboration of veterinarians – pathologists and virologists, ornithologists, ecologists, biologists, mathematics and other scientist together with high through put biotechnology applications, has offered a progress to understand the HPAI H5 viruses epidemiology and evolution, still pose a risk for animal and human health globally.

Furthermore, understanding the ecology of avian influenza, the evolution in wild birds, is the key maybe to a global health framework, where the evidence based and scientific driven improvement in food security, animal health and socioeconomic development to manage mitigation of the infectious disease.

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Appendix

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List of Abbreviations

a. AIV: Avian Influenza virus
b. AGID: Agar Gel Immunodiffusion Assay
c. CNS: Central nervous system
d. ECDC: European Centre for Disease Prevention and Control
e. EFSA: European Food safety Authority
f. ELISA: Enzyme-linked immunoassay
g. E.U.: European Union
h. FAO: Food and Agriculture Organisation
i. HPAI: High pathogenic avian influenza
j. IVPI: Intravenous Pathogenicity Index
k. LPAI: Low pathogenic avian influenza
l. OIE: World organisation for Animal Health
m. PCR: Polymerase Chain Reaction
n. SAN: Specific Antibody Negative eggs
o. SPF: Specific Pathogen Free
p. WHO: World Health Organisation