Original Research Article

Comparative analysis of Human and Avian Influenza Virus in Greece (2002-2011)

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The aim of this study was to evaluate and compare the epidemiological data recorded by the surveillance programmes applied in Greece, during the decade 2002-2011, by both medical and veterinary authorities. Sentinel surveillance system was used to analyze influenza virus cases in humans, while passive surveillance system was initiated by proper directive in 2005 onwards. Data retrieved by competent authories showed that the majority of human cases were both of type A and B, whereas type A samples were identified of subtype H1N1 and H3N2. Among the animal specimens tested for this specific study period, only 35 proved to be positive in 2006. All except for one positive animal case were identified as of subtype Highly Pathogenic Avian Influenza Virus H5N1, except for one which was of subtype Low Pathogenic Avian Influenza Virus H6N2. Almost 45% of humans were of paediatric population and 100% of animals were wild bird species. No corellation of influenza types between humans and animals was observed, in case of Greece.

Key words: Avian influenza, Greece, epidemiology

INTRODUCTION

Avian Influenza (AI) virus belongs to Orthomyxoviridae family and is divided in three genera: A, B and C. Genera A is known to affect avian species, whereas isolation of the virus or sequencing of its genome is required, as the virus provokes a variety of symptoms, which differ according to the host and its immune status, the virus' strain and many other coexistent parameters (World Organisation For Animal Health 2015). AI is hosted in wild aquatic birds, causing mild or subclinical symptoms of infection (Alexander 2007). Poultry are more susceptible to such infections, whereas mortality depends on the pathogenicity of the virus. This is of great importance, as avian influenza virus can potentially lead to a human pandemic (Kyriakis et al., 2011) (Fiebig et al., 2011).

Virions of AI contain as nucleic acid 8 molecules of single-stranded ribonucleic-acid (RNA). This genetic material is covered by a capsid (protective coat) created by protein M (matrix, which consists of M1, that binds to viral RNA and M2, that acts as ion channel) and the surface glyco-proteins hemagglutinin (H) and neuraminidase (N) (Abolnik 2014). There are sixteen different Hs (1-16) and nine different Ns (1-9). Humans have H:1,2,3,5,9 and N: 1,2, while poultry have all the types of H and N (Tong et al., 2012) (Tong et al., 2013).

Avian Influenza A viruses are divided in two separated groups, depending on the ability to cause disease:1) *Highly Pathogenic Avian Influenza viruses*, which can cause extremely severe disease and is characterized by general infection of affected poultry (Lee ja Song 2013). Although most of H5 and H7 subtypes isolated till to date have been of low virulence, they are classified as notifiable avian influenza virus, due to the risk to become virulent by mutations. The mortality rates range between 50 to 100% (World Assemby Delegates of OIE 2009). Differential diagnosis should be held among HPAI viruses fowl plague, infectious laryngotrachiitis, acute toxications and other diseases, 2) *Low Pathogenic Avian Influenza viruses*, which, mainly, cause mild respiratory symptoms to poultry chickens, while no other risk factors or infections exist. Clinical signs can vary from no to many and severe, that could also lead to high monimals, with different sensitiveness degree, low contact rates and, relatively, low

population density (Ullah et al., 2014) (Post et al., 2013) (Peng et al., 2013) (Takekawa et al., 2010).

In the last decade, humanity has faced several severe pandemics. The first one named "Spanish Influenza" (H1N1) was dated in 1918 and costed more than twenty or fourty million people's lives (Lazzari ja Stöhr 2004) (Swayne 2009). The second pandemic came up fourty years later, in 1957 and named "Asiatic influenza" (H2N2), where more than a million people died (Lazzari ja Stöhr 2004). In 1968, a third pandemic arrived with the name "Hong Kong Influenza" (H3N2) and affected more than 700.000 people. A great outbreak took place, also, in 1977 (H3N2, H1N1). Humans were infected by low pathogenic AI (H9N2) in China in 1998 and Hong Kong in 1999 and 2003 (Swayne, 2009). Low pathogenic AI virus (H7N2) affected people in United States of America, in 2002 and 2003, and in United Kingdom in 2007, while other AI subtypes, such as H7N3 and H10N7 affected population of United Kingdom in 2006 and Egypt in 2004, respectively (Jonges et al., 2011). Sometimes, epidemics come about pandemics, affecting more susceptible population parts, such as children, elderly and immunosupressed people (Katz, 2003) (Lang, 2013).

All these low pathogenic viruses can cause mild symptoms to humans, while AI subtypes H5 and H7 can effortlessly undergo mutations, which convert them to Highly Pathogenic AI viruses that can affect poultry farms (Lang 2013).

Direct transmission to humans by poultry was believed to be unfeasible, untill 1997, when the outbreak of Hong Kong took place (Sims ja Peiris 2013). Humans can be affected when they come in contact with affected poultry, their faeces, nasal excreta and contaminated dust. AI virus can lead to human pandemic when the viruses (i) can proliferate into humans, (ii) possess the correspondent hemagglutinin subtype and (iii) can be transferred from human to human. Humans have only few receptors for AI virus and are, therefore, infected rarely, compared to other species, such as pigs, which are reservoirs of different types of viruses. So, when human and avian influenza viruses are met, exchange redeployment takes place and a new viral type is created. This new type can be more virulent and may be transferred among non-immunized humans very easily (Kang et al., 2015).

The aim of this study is to combine and compare comprehensive epidemiologic data collected by medical and veterinary control authorities during 2002 to 2011, in order to identify any potential correlation of the influenza types infected humans and animals in Greece. To our knowledge this is the first study to address possible corellation of human and animal influenza types in mediterranean countries.

MATERIALS AND METHODS

Before 2005, Greek authorities just applied a monitoring programme of AI virus. 2005 onwards, as recommended by guidelines of World Health Organization (WHO) and World Organization for Animal Health and indicated by the European legislation, applied in all European countries-members (Council Directive 2005/94/EC), Greek Ministry of Rural Development and Food has established Epidemiological Surveillance Program for Avian Influenza Virus, which should be carried out by National Veterinary Laboratories and National Reference Laboratory for Avian Influenza Virus. According to this program, fifty five Local Disease Control Centres, each one in each perfecture, were responsible for recording and sampling any kind of poultry farms and wild animals. In compliance with the guidelines provided, samples taken from dead birds should contain faeces or cloacal swabs and oropharyngeal swabs, while concerning live birds, samples should contain both cloacal and oropharyngeal swabs that are to be taken gently. Infected tissues, such as, intestine, brain, trachea, lungs, liver, spleen or others can also be sampled and tested. Samples can be pooled together, while faecal samples should be separated. Not to mention, that swabs taken from different sites or tissues shouldn't be mixed. Once found a positive sample, this was inoculated into the allantoic cavity of 9 to 11 days old embryonating chicken eggs, in order to test the haemmagglutinating activity in all eggs. Haemmagglutination and neuraminidase inhibition test were, also, prepared.

As far as poultry animal is concerned, legislation imposes sampling of the hall animal population, when the flock size is smaller than 20, while 25% or more animals should be sampled, when the flock size is greater than 20 (99% probability of detecting a positive sample). Blood samples should be put in appropriate venojects, without anticoagulating agent. They are centrifuged and the serum taken is tested for detection of the antibodies to specific antigen.

All samples used in this study were sent to and tested by National Reference Laboratory for Avian Influenza Virus (Figure 1). Identification of avian influenza virus imposes swab sampling. Swabs taken by individual birds, should be put in sufficient antibiotic mean, for more than two hours in room temperature, in a way to ensure that all are plunged deeply. The antibiotic means used may vary among laboratories. The one used for the data of our study was of the following constitution: 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 0,25 mg/ml gentamycin and 5000 IU/ml mycostatine in buffer solution phosphate buffered saline. The standard operating protocols (SOPs) used in National Reference Laboratory for Avian Influenza Virus were: Matrix-, N1-, H5- and H7-gene with real time Taqman RT-PCR, based on the protocols used by Community Reference Laboratory (CRL), Veterinary Laboratories Agency (VLA). Samples proved to be positive were also identified by CRL, given the fact that not all of H- and N- genes were prepared in National Reference Laboratory for Avian Influenza Virus.

Poultry farm animals were, also, tested, mainly, by blood samping. All blood samples were tested by serological testing methods, which indicates prior exposure to the
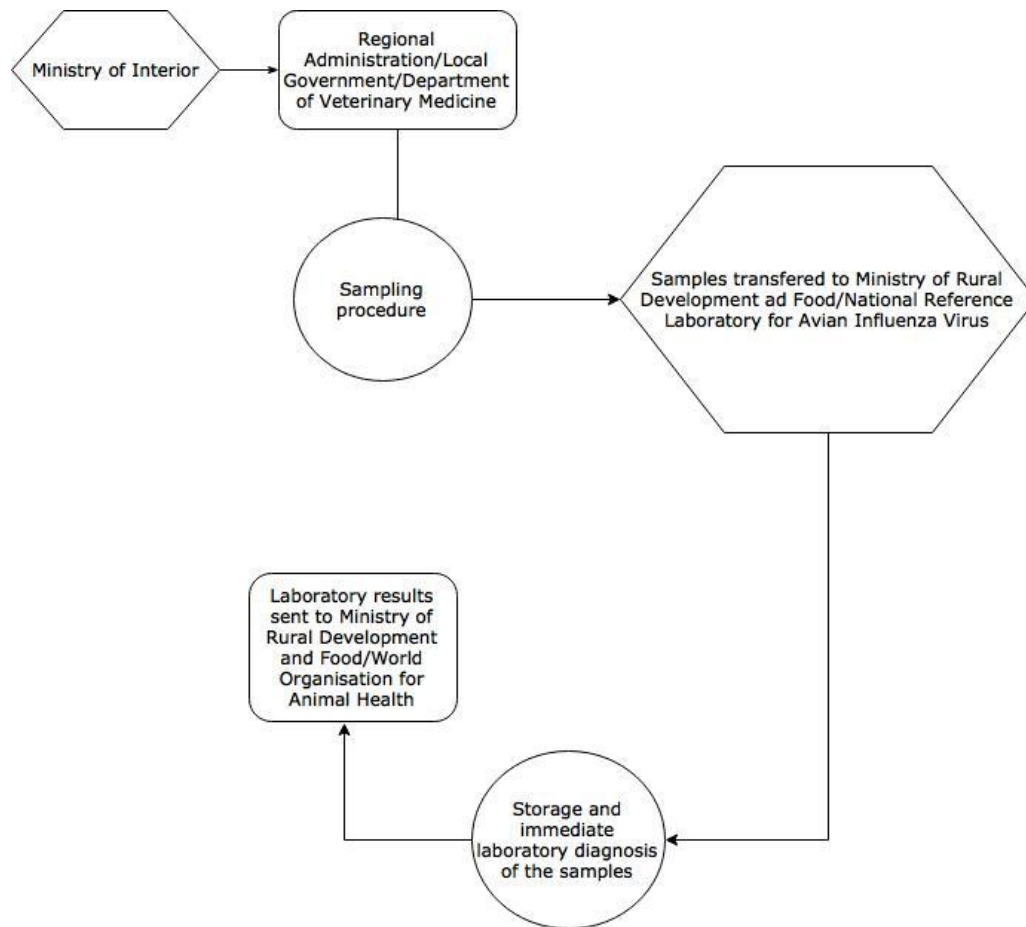
Figure 1: Avian Influenza Virus Surveillance process in Greece

specific virus by detecting antibodies to antigens to all influenza A viruses, in blood samples. The routine serological test applied was Enzyme Linked Immunosorbent Assay (ELISA). The commercially available ELISA test used were only for subtypes H5 and H7, as these were regarded of economic importance and have been classified as notifiable avian influenza virus (World Assemby Delegates of OIE 2009). There are two types of ELISA used: the one for dependent species (indirect) and one for independent species (competitive). Alternatively, Haemagglutination or Haemmagglutination inhibition tests are, also, proposed by OIE (OIE Terrestrial Manual). Once a positive (true or false) result appears in ELISA, this is followed by Haemagglutination and Haemmagglutination inhibition tests. This serological method can determine every hammagglutinin subtype, while using homologous or closely related antigen. All samples collected were also accompanied by specific brochure, where perfecture, species, type of farm, name of the owner, flock size, flock age, medication and vaccination history of the farm or animal were referred, in order to fullfill the profile of the samples taken.

In year 2002 almost 2040 blood samples were tested. All samples were of farm poultry animals and aged between 2 days to 8 months old. In year 2003, 10774 blood samples were collected and tested. They were, also, farm animals and aged between 1 day to 17 months old. In year 2004, 2699 farm poultry animals were tested via blood sampling and they were 1 day to 16 months old. In 2005, 3913 blood samples of poultry farm animals were collected. The animals' age was between 1 day to 2 years old. Till this chronical point, all samples received were tested via serological method, ELISA, which has already been described. Concerning laying hens, broilers, chicks, free-range broilers and ducks, indirect ELISA method has been used, while concerning samples from turkeys, competitive ELISA was the selection method. Since 2006, when the "hurricane" began to spread, molecular methods (real time rt-PCR) were used for swab (oropharyngeal and cloacal) samples and about 2156 samples were tested. 1920 were poultry and domestic farm animals, aged between Those were mixed farm poulrty and wild birds animals, aged between 1 day to 5 years old. Some of the individuals captured and tested were of unknown age and were identified by appropriate scientists. Voluntary organisations for the hospitalization of wild animals tried to offer to this direction, by putting identification rings to any wild animals captured, so that they could know more

Table 1. Animal surveillance 2002-2011

Years	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Number of samples	2040	10774	2699	3913	2156	5931	3992	2771	2708	4500
Type of species	Farm poultry	Farm poultry	Farm poultry	Farm poultry	Mixed (Farm poultry>wild birds)	Mixed (Wild birds>farm poultry)	Mixed	Mixed	Mixed	Mixed (Farm poultry>wild birds)
Type of samples	Blood	Blood	Blood	Blood	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
Analysis technique	ELISA	ELISA	ELISA	ELISA	Mixed (serological and molecular)	Mixed (serological and molecular)	Mixed (serological and molecular)	Mixed (serological and molecular)	Mixed (serological and molecular)	Mixed (serological and molecular)
Age range	2-240 days	1-510 days	1-480 days	1-720 days	1-1800 days	1-1440 days	2-unknown days	2-720 days	1-720 days	1-450 days

about the spread of the virus and the measures to be taken. Most of poultry farm animals were tested via blood sampling with ELISA method and individually wild animals were tested via swabbing. All the samples, since then, were accompanied by a specific document, where precious information for the correspondong samples was available. The document recommended by Ministry of Rural Development and Food contains information like: A) Concerning the sample code: 1) the identification number of the animal (where available), 2) the age of the animal, B) Concerning the sample's type: 3) the sample type (cloacal swab, fresh excreta, trachea's/pharynx; swab, tissue, blood, or other), 4) the date of sampling procedure, 5) the productive type of the animal (laying hen, free-range broiler chicken, organic broiler, day-old chick, scientific names of wild birds, etc), C) Concerning the sample's geographical data: 6) the area of sampling, 7) the city or town, 8) the longitude, 9) the latitude, 10) the area type (area free of restrictive measures, checkpoint zone, investigation zone, precaution zone, zone under surveillance, Area A and B, concerning Avian Influenza Virus), D) Concerning the animal's origin:

11) the animal's condition: alive, trapped or injured, hunted, sick or found dead, 12) the flock size, 13) the country of origin, 14) the number and issuing principle of health certificate and 14) the code of the farm. In 2007, 5931 poultry farm and wild animals were tested and aged beween 1 day to 4 years old. Most of them were wild animals, because of the public's view that those were the aim of the "evil" that initialised them to capture more and more wild animals and test them for avian influenza virus. In 2008, 3992, also, mixed samples were tested. They were estimated between 2 days and unknown years old. In 2009, 2771 of all kind of samples were taken and tested. They were of 2 days to 2 years old. In 2010, the samples' number met 2708, while ages were between 1 day to 2 years old. In 2011, 4500 of all kind of samples were taken. Most of the samples were poultry farm animals imported from foreign countries, mainly Germany, Poland, Great Britain, Czech Republic, Italy and Hungary and were aged 1 day to 15 months old (Table 1).

Humans' cases of the virus was surveyed by Sentinel surveillance network and applied by health workers or health facilities operators, like private physicians (i.e. general practitioners, pediatricians) and small hospital units, respectively, during periods of influenza surveillance (October to May of each year). Inclusion criteria comprised persons of different ages with clinical evidence (defined as any infection of respiratory tract), with or without fever (≥38.5°C) over duration of ≤72 hours. The data collected for each patient included in the sampling procedure were patient's name, age, sex, vaccination profile, duration of infection at consultation time and presence of symptoms, like temperature, sore throat, nasal secretion, cough and gastrointestinal symptoms. Throat and/or nasal samples were collected and sent for laboratory diagnosis at 4°C in minimal essential medium enriched with 1% bovine serum albumin to reference public health laboratory. The specimens were inoculated, incubated and sublected to direct fluorescence assay with monoclonal antibodies against influenza of A and B genera. All influenza A positive samples were subtyped by indirect fluorescence assay with specific antibodies (Figure 2).

Based on Hellenic Centre for Disease Control and Prevention and as far as human samples is concerned,
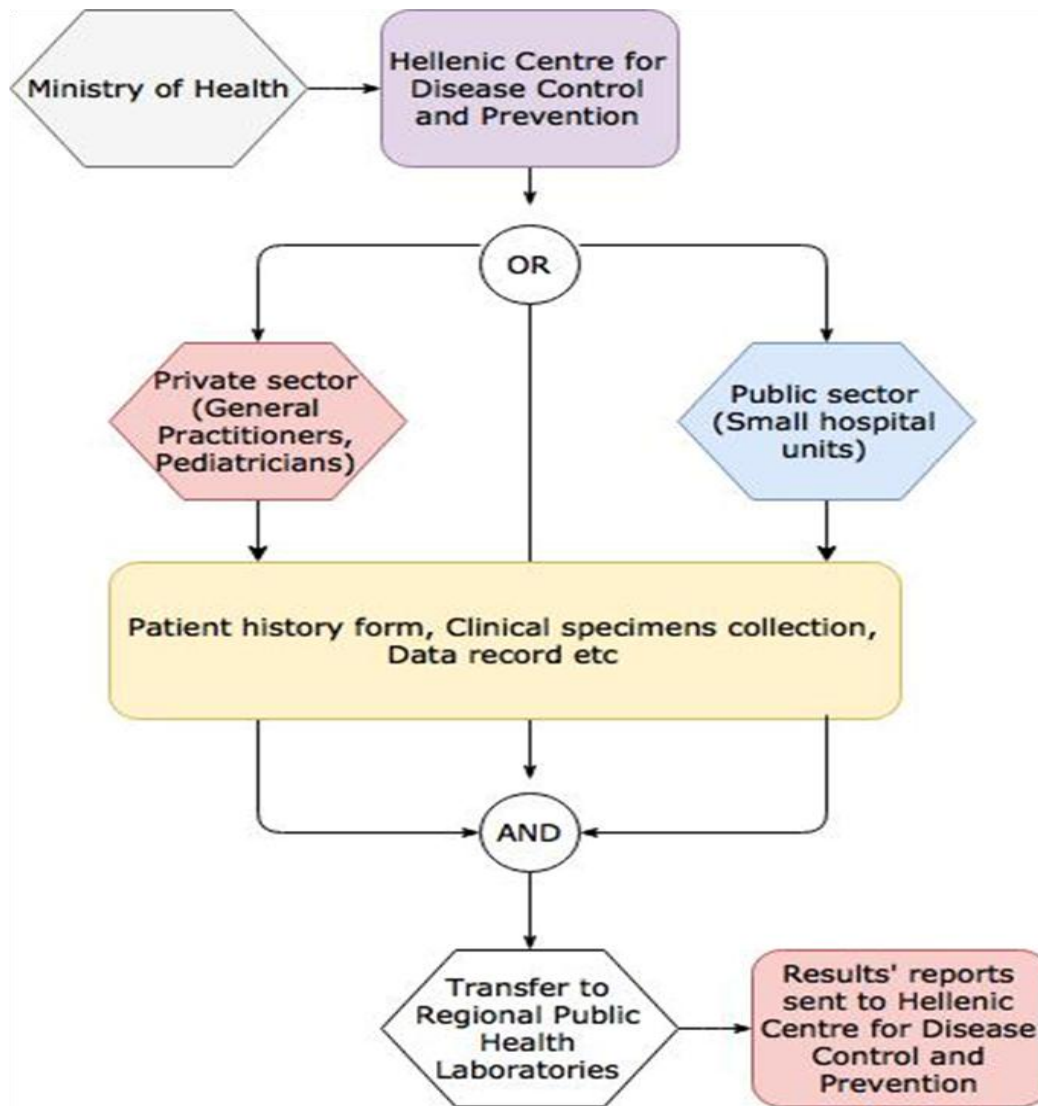
Figure 2: Influenza Sentinel surveillance system process in Greece

in 2003 105 samples were collected. In 2004, 226 samples were tested. In 2005 and 2006, 433 and 386 samples were, respectively, taken and tested. In 2007 , the number of specimens tested reached 624. In 2008, 699 were tested. In 2009, 521 clinical samples were collected nad tested, while in 2010 clinical samples were only 71. In 2011, the samples were 355 (Figure 3).

RESULTS

Animals

A total of 35 simple animal cases were identified as positive for Avian Influenza Virus. All of them, except for one, were encountered in year 2006, near sea-, river- and lake-sided areas and only in individual dead wild birds, whereas 30 were mute swans (*Cygnus olor)*, 1 was

Northern shoveler (*Anas clypeata*), 1 whooper swan (*Cygnus cygnus*), 1 great cormorant (*Phalacrocorax carbo*) and 1 wild goose. All samples tested and proved to be positive in ELISA and Haemagglutination/ Haemagglutination inhibition test and/or real-time rt-PCR, were also, sent to European Veterinary Laboratories Agency (VLA) of Weybridge (United Kingdom) for further confirmation. All of the samples that found to be positive in National Reference Laboratory for Avian Influenza Virus, were identified also, as positive and were of type A and subtype Highly Pathogenic Avian Influenza Virus H5N1, except for the one Northern shoveler (*Anas clypeata*) whose subtype was Low Pathogenic Avian Influenza Virus H6N2. All these animals were found dead near the areas, were collected and sent to the laboratory for sampling and testing. The only case proved to be positive, other year than 2006, was in 2010, when a poultry farm sample of a duck that was imported by Hungary and was alive
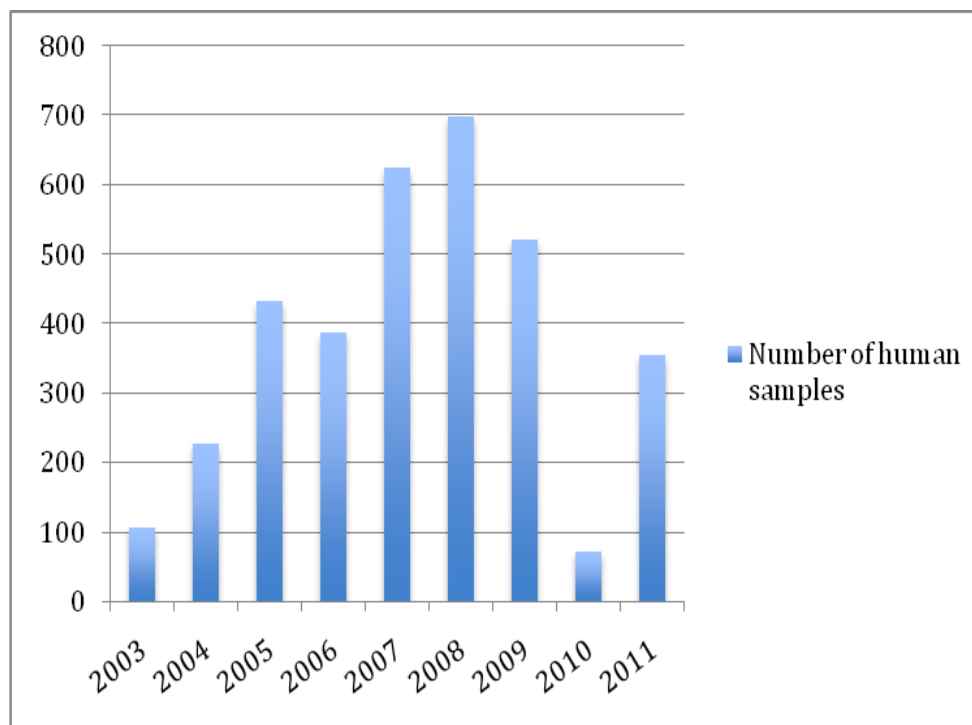
Figure 3: Human clinical specimens test per year 2003-2011

(trapped or injured) in area Megara Attiki, where no water ponds were met (Table 2).

In this study, the prevalence of AI was zero for all years of the study, except for 2006 and 2010, that was calculated to be 0.0154 (34/5199) and 0.00037 (1/2708), respectively.

Humans

In 2003, the human samples tested proved to be positive were 62 out of 105. 18 of these were of type A, while 17 of these were of subtype H3N2 and 1 of H1N1. No sample of type B was detected. In 2004, 226 samples were tested, while 81 of these were positive. 75 were of type A, three of type B and three of unknown type. Type A positive samples were of H3N2 and H1N1 subtype. In 2005, 40 samples proved to be positive. 37 of the 40 were of type B and only 3 of type A and subtype H3N2. In 2007 311 out of 624 samples were positive. 262 out of 311 were of A type and subtype H3N2 and 49 of the positives were of B type. In 2008, 215 out of 699 clinical samples collected and tested were positive. 145 of these were of type A, all of which were of subtype H1N1. The rest 70 positive samples were of type B. Concerning the year of 2009, 297 out of 521 samples were positive. In 132 of the positive cases A type influenza virus was isolated. All type A cases were of H3N2 subtype. 165 of positive samples were of type B. In 2010, only 6 out of 71 samples were positive. All were of type A: 5 of H1N1 (new) and one of H3N2. In 2011, 187 out of 355 clinical samples were positive. All those 187

positive samples were of type A: 185 of H1N1(new) and 2 of H3N2 subtype, while 9 positive samples were of type B (Table 3) (Figure 4) (Figure 5).

DISCUSSION

To our knowledge, this is the first comparative analysis attempted for both humans and animals in Greece. Our findings of temporal peaks in influenza virus activity in Greece are consistent with data reported from Vietnam and other South East Asian countries (Finkelman et al., 2007), while avian influenza virus seasonal peak was, also consistent with studies conducted in Germany, Thailand, Indonesia and other regions (Liu et al., 2015) (Probst et al., 2012) (Loth et al., 2011) (Aditama et al., 2011). No correlation observed between influenza types recorded in humans and animals in Greece.

Both animal and human populations used for this study are supposed to be "open", as during the period of the study, human or animal populations could be added or removed. It is estimated that both populations are of "steady state" or "stationary", as populations added and removed seem to be almost equal.

In contrast to the ongoing human infections occured, albeit limited cases compared to the total human population, avian influenza virus outbreaks that struck animals in Greece were recorded only in year 2006 of and infected only 35 animals. The distribution of the human population of this study shows that almost 45% correspond to children. But this is not surprising, as it is

Table 2. Animal positive specimens data

Laboratory Test	Number of samples	SAMPLE CODE		SAMPLE TYPE			SAMPLE'S GEOGRAPHICAL DATA					BIRD ORIGIN	RESULT
Laboratory Test	Number of samples	Perfecture	Year	1. Cloaca's swab	Date of Sampling	Bird Type	Area	City/Town	Longitude	Latitude	1. Area free of restrictive measures	1. Alive (trapped, injured)	
				2.Fresh excreta							2. Checkpoint zone	2. Hunted	
				3. Trachea's/ Pharynx' swab							3. Investigation zone	3. Sick	
				4. Tissue							4. Precaution zone for Avian Influenza	4. Found dead	
				5. Blood							5. Surveillance zone for Avian Influenza		
				6. Other							6. Area A		
											7. Area B		
											8. High risk area		
rt RT-PCR/106	1	Veterinary Laboratory of Katerini	2006	6	29-01-06	Cygnus olor		Pieria	40° 15' 56.84''	22° 35' 50.71''	1	4	H5N1
rt RT-PCR/125	1	Thessaloniki	2006	6	31/01/06	Cygnus olor	Stavros	Thessaloniki	40°39' 52.35''	23°42'0 5.83''	1	4	H5N1
rt RT-PCR/125	1	Thessaloniki	2006	6	31/01/06	Cygnus olor	Paralimni Giannitsa	Pella	40°45'0 5.09''	22°27'1 0.95''	1	4	H5N1
rt RT-PCR/125	1	Thessaloniki	2006	6	31/01/06	Cygnus olor	Thasos	Kavala	40°43'0 9.47''	24°40'0 0.46''	1	4	H5N1
rt RT-PCR/128	1	Thessaloniki	2006	6	01/02/06	Cygnus olor	Nei Epivates	Thessaloniki	40°30'3 6.00''	22°57'5 3.46''	1	4	H5N1
rt RT-PCR/165	1	Thessaloniki	2006	6	02/02/06	Cygnus olor	Thessalonik i Coast	Thessaloniki	40°38'2 1.66''	22°56'4 0.59''	1	4	H5N1
rt RT-PCR/166	1	Thessaloniki	2006	6	01/02/06	Cygnus olor	Asprovalta	Thessaloniki	40°43'1 7.62''	23°42'2 5.92''	1	4	H5N1
rt RT-PCR/167	1	ILPAN Athens	2006	2	03/02/06	Anser albifrons	Kymi		38°37'5 7.31''	24°06'1 3.17''	1	4	H5N1
rt RT-PCR/188	2	Rhodope	2006	3	04/02/06	Cygnus olor	Xerolimni Fanari	Rhodope	40°57'3 4.37''	25°07'5 0.24''	1	4	H5N1
rt RT-PCR/208	1	Kassandra Chalkidiki	2006	6	10/02/06	Cygnus olor	Polychrono Kryopigi	Chalkidiki	40°00'5 9.46''	23°31'4 0.83''	1	4	H5N1

Table 2. Contin.

|													
rt RT-PCR/222	1	Katerini	2006	3	10/02/06	Cygnus olor	Port of coast	Pieria	40°16'17.34''	22°30'31.18''	1		H5N1
rt RT-PCR/222	1	Katerini	2006	3	10/02/06	Cygnus olor	Makrygialos	Pieria	40°24'55.40''	22°36'11.11''	1		H5N1
rt RT-PCR/230	1	Thessaloniki	2006	6	12/02/06	Cygnus olor	Stavros	Thessaloniki	40°39'52.35''	23°42'05.83''	1	4	H5N1
rt RT-PCR/262	1	Giannitsa	2006	6	12/02/06	Cygnus olor	Loudias River, Nea Pella	Pella	40°37'59.89''	22°28'26.22''	1	4	H5N1
rt RT-PCR/266	1	Thessaloniki	2006	6	13/02/06	Cygnus olor	Paliouras Epanomi	Thessaloniki	40°24'15.91''	22°53'52.65''	1	4	H5N1
rt RT-PCR/288	2	Ierissos	2006	6	13/02/06	Cygnus olor	Saltpits Ammouliani	Thessaloniki	40°19'55.97''	23°55'17.74''	1	4	H5N1
rt RT-PCR/346	1	Veria	2006	6	16/02/06	Cygnus olor	Area 66 Apostolos Pavlos	Imathia	40°33'45.40''	22°15'44.09''	1	4	H5N1
rt RT-PCR/350	1	Feres	2006	6	15/02/06	Cygnus olor	Feres	Evros	40°53'35.91''	26°10'25.45''	1	4	H5N1
rt RT-PCR/351	2	Xanthi	2006	1	15/02/06	Cygnus olor	Porto Lagos Lagoon	Thessaloniki	41°00'40.82''	25°08'45.31''	1	4	H5N1
rt RT-PCR/391	2	Rhodope	2006	6	10/02/06	Cygnus olor	Lake of Xirolimni	Rhodope	41°30'15.94''	24°42'51.45''	1	4	H5N1
rt RT-PCR/391	1	Rhodope	2006	6	12/02/06	Phalacrocorax carbo	Fanari	Rhodope	40°57'34.37''	25°07'50.24''	1	4	H5N1
rt RT-PCR/403	1	Evros	2006	4	16/02/06	Cygnus cygnus	Metaxa	Evros	41°34'27.24''	26°31'49.78''	1	4	H5N1
rt RT-PCR/429	1	Didymotiho	2006	6	21/02/06	Cygnus olor	Pythio Didymotiho	Evros	41°22'52.73''	26°36'36.55''	1	4	H5N1
rt RT-PCR/435	1	Orestiada	2006	6	23/02/06	Cygnus olor	Evros River	Evros	41°00'51.67''	26°14'24.19''	1	4	H5N1
rt RT-PCR/436	2	Veria	2006	6	23/02/06	Cygnus olor	Agios Georgios	Imathia	40°36'15.26''	22°11'30.96''	1	4	H5N1
rt RT-PCR/446	1	Kavala	2006	6	27/02/06	Cygnus olor	Iraklitsa	Kavala	40°51'56.59''	24°21'10.79''	1	4	H5N1
rt RT-PCR/455	1	Thessaloniki	2006	6	26/02/06	Cygnus olor	Saltpits Epanomi	Thessaloniki	40°25'31.69''	22°55'43.17''	1	4	H5N1
rt RT-PCR/492	1	Thessaloniki	2006	6	04/03/06	Cygnus olor	Palioura Saktpits Epanomi	Thessaloniki	40°24'15.91''	22°53'52.65''	1	4	H5N1
HA/892	1	Chalkida	2006	6	15/11/06	Anas clypeata	Anthili/Sperhios River		38° 51' 00''	22° 28' 50''	1		H6N2
rt RT-PCR/122	1	ILPAN Athens	2010	1	22/05/2010	Duck	Megara	Attiki			1	1	H5N1
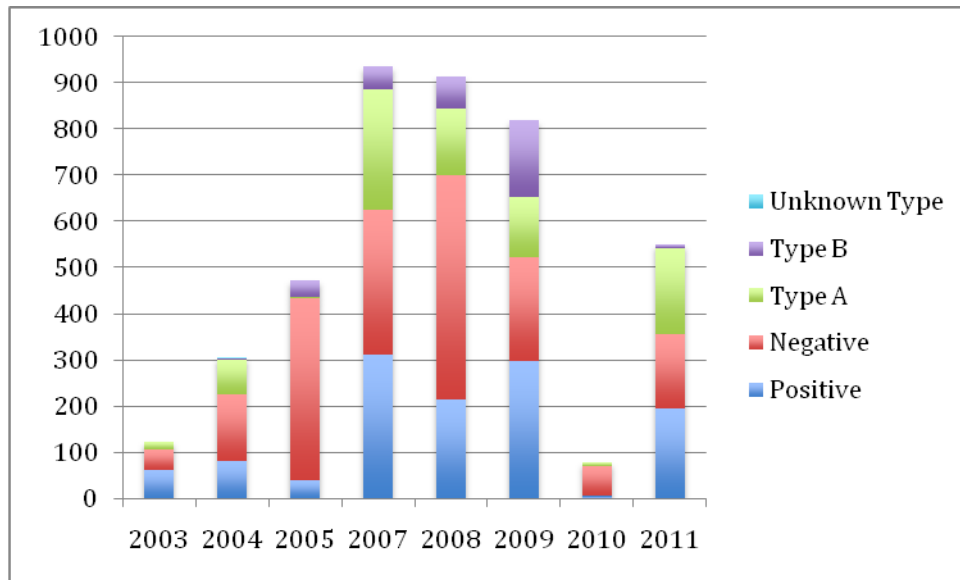
Figure 4: Human positive distribution results upon year
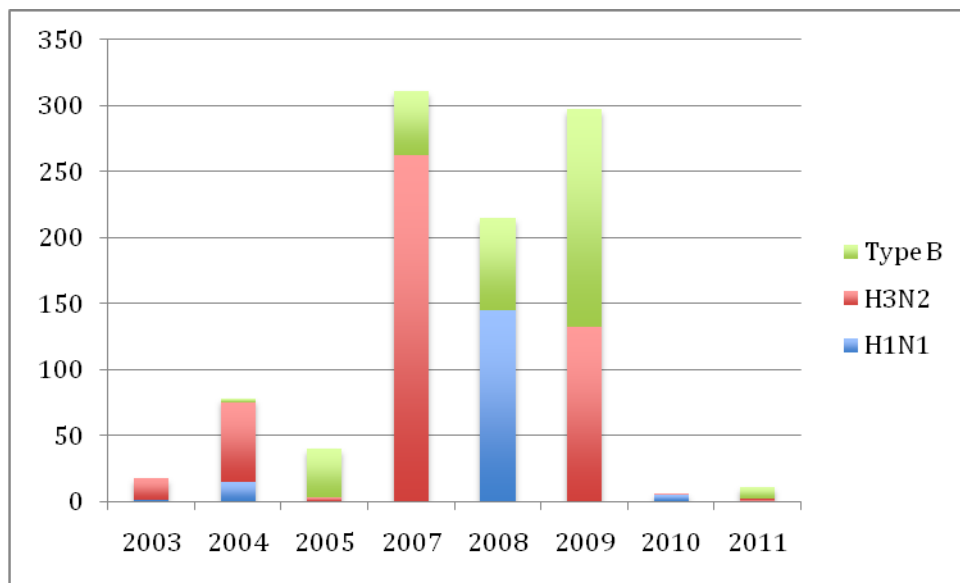

Figure 5: Human positive subtype distribution upon year

widely accepted that paediatric population is the main reservoir of respiratory viruses like influenza one (Bennett et al., 2014) (Munoz 2002). Concerning animals, all avian influenza cases were individual wild birds, which is also not unexpected as wild birds are considered to be primary avian influenza virus reservoir (Olsen et al., 2006) (Suarez 2008) (Śmietanka et al., 2014). Data from surveillance period indicated that most of human positive samples were of type A and subtype H1N1 and H3N2, while type B influenza cases existed in lesser percentage. Overall, no temporal overlap between influenza A and influenza B peaks was observed. Both influenza type co-circulated. H3N2 and H1N1 A viruses predominated in our study

period with little overlap, as observed in temperate countries (Finkelman et al., 2007). Avian influenza viruses circulated throughout Greek animal population were all of type Highly Paathogenic Avian Influenza Virus H5N1, except of one Low Pathogenic Avian Influenza Virus H6N2. Data from national surveillance system, also, indicated that each year of study period, except for 2003, had two distinct peaks in influenza virus circulation among Greek population, unlike other countries' temperate climates (Nguyen et al., 2009). Peak of avian influenza virus cases was observed in January and February. This temporal peak coincided with lower water and environment temperatures and rainy periods in Greece. For that reason

higher probability of avian influenza virus clinical cases at this certain period was expected (Nunes et al., 2005).

Sentinel surveillance system deliberately involves a limited network of carefully selected operators, unlike other passive surveillance systems. Sample collection in human cases is made based on clinical criteria of influenza-like illness (Navarro-Marí et al., 2005), unlike sample collection in animals, which is determined on percentage basis, depending on various factors (Anderson et al., 2010). The significant increase of AI prevalence in animals, in 2006, can be attributed to various factors, like the surveillance system imposed by WHO Directive in 2005, that mandated the surveillance and sampling of animal populations, the control and prevention measures applied and the comprehensive virologic analysis of the disease of official public health reference laboratories.

Starick et al. also recorded Highly pathogenic Avian Influenza Virus in Germany for years 2006 and 2007, suggesting separate introductions of H5N1 subtype of closely related H5N1 viruses, originating from Southern and Central Russis (Starick et al., 2008)Nguyen et al., analysing the national influenza surveillance system applied in Vietnam agreed with our results that influenza virus were detected year round, but with higher influenza axctivity peaks in cooler and rainy periods of the year (Nguyen et al., 2009). Jiang et al. addresed laboratory confirmed Highly Pathogenic Avian Influenza Virus H5N1, H5N6 and H7N9 in humans in mainland China, after potential exposure of those human to domestic and retail animals, visit to live poultry markets or direct contact to poultry (Jiang et al., 2017).

Limitations

There are some limitations of our study. Firstly, our epidemiological analysis of animal species originate from National Reference Laboratory of Greece, which is responsible to test all positive suspicious samples but not all of the samples taken in Greece. This means that sample size of the whole country may vary, although positive results remain the same. Secondly, our analysis of the epidemiology of human cases is based on single case notifications declared by individual collaborating physicians and it is possible that some human cases have occurred and were not laboratory confirmed, either because of lack of clinical suspicion or lack of access to physician or/and laboratory testing in some areas. Last but not least, only a small number of human cases have been reported to the requested time period of our study, limiting our ability to characterize the differences between influenza infections in human and animal populations.

Future directions

As literature suggests that Highly Pathogenic Avian Influenza Virus human cases were most probably initiated by Low Pathogenic Avian Influenza Virus cases in poultry, our study suggests that increased awareness by both veterinary and medical authories is needed. The laboratory confirmed human cases around the world alert us and it remains to be seen whether avian influenza virus will continue to circulate among poultry and cause sporadic infections of human infections in future years. Continued surveillance of both human and animal infections remains an absolutely necessary component of pandemic preparedness, and further investigations are needed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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