UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

WILD BIRDS AS A SOURCE OF SELECTED EMERGING AND RE-EMERGING PATHOGENS: THE WEST NILE VIRUS EXAMPLE IN GREECE

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Georgios Valiakos, MSc

Supervisor: Charalambos Billinis, Professor

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Postgraduate Student:
Georgios Valiakos, DVM, MSc

Supervisor:

Charalambos Billinis, Professor, Faculty of Veterinary Science, University of Thessaly

Advisory Committee:

Liljana Petrovska, Animal Health and Veterinary Laboratories Agency

Vassiliki Spyrou, Associate Professor, Department of Animal Production, Technological Education Institute of Thessaly

Examination Committee:

Angeliki Rodi-Burriel, Professor, Faculty of Veterinary Science, University of Thessaly

Zissis Mamuris, Professor, Department of Biochemistry and Biotechnology, University of Thessaly

Christos Hadjichristodoulou, Professor, Faculty of Medicine, University of Thessaly

Konstantinos Koutoulis, Lecturer, Faculty of Veterinary Science, University of Thessaly

Liljana Petrovska, Animal Health and Veterinary Laboratories Agency

Vassiliki Spyrou, Associate Professor, Department of Animal Production, Technological Education Institute of Thessaly

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All samples used in this PhD thesis represent opportunistic samples that were collected for purposes other than the WildTech project. With regard to ethical considerations, all activities were performed strictly according to the European and Greek Guidelines.

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George Valiakos
University of Thessaly
July 2015

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INTRODUCTION

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INTRODUCTION

Despite improvements in medical science over the past decades, infectious diseases remain major cause of mortality throughout the developed and developing world. A large number of causative agents (61% in 313 different genera, of the 1,415 identified human pathogens) are zoonotic and infect multiple animal species (Taylor et al. 2001). Approximately 75% of all diseases, including zoonoses, which have emerged in the last few decades are of wildlife origin (Bengis et al. 2004).

Various wild avian species are considered to have major epidemiological role in the introduction and establishment of various pathogens in remote areas. Migratory avian species travel long distances in a few days, being able to transport pathogens in new areas and thus there is increasing public health concern regarding their role in spread of diseases. Moreover, resident wild avian species may contribute in the local establishment and amplification of pathogens that cause disease in humans and livestock animals. More than 50 pathogens for which wild birds can serve as reservoirs, mechanical vectors, or both, have been described in recent literature (Tsiodras et al. 2008).

One of the most important pathogens linked to wild birds in recent years is West Nile virus, a mosquito-borne flavivirus that has become a major public health concern in the last two decades, due to the increased disease outbreaks worldwide. Humans and other mammals, particularly horses, are alternative hosts of WNV; main route of infection is through the bite of an infected mosquito. While most human infections remain asymptomatic, West Nile fever develops in ≈20% of infected persons and West Nile neuroinvasive disease in <1% (Mostashari et al. 2001). Nonetheless, horses and humans develop viremia levels of low magnitude (<10^5 PFU/ml) and short duration, mainly insufficient to infect mosquitoes and thus do not serve as amplifying hosts for WNV in nature (Bunning et al. 2002). On the contrary, various avian species, both migratory and sedentary develop viremia levels sufficient to infect feeding mosquitoes (Komar et al. 2003). Hence, WNV is maintained in an enzootic cycle with wild and domestic birds acting as the main amplifying hosts and ornithophilic mosquitoes, especially of the Culex species, the main vectors. Moreover, local movements of resident birds and long-range travel of migratory

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birds may both contribute to the spread of WNV (Rappole et al. 2000, Komar et al. 2005).

In the last decade human cases of West Nile fever were reported in several EU and bordering countries (Romania, Italy, Hungary, Spain, Turkey, Israel and Russia). Since 2010, a major WNV outbreak of human infections took place in Greece. From 2010 to 2014 more than 624 laboratory-confirmed cases and 73 deaths were caused by the virus (Table 1).

Year	Laboratory-confirmed human cases	Deaths	%	Area of Effect
2010	262	35	17%	Northern Greece (Central Macedonia)
2011	100	9	12%	South Dispersion to Thessaly and Continental Greece
2012	161	18	17%	Area of Attica and North-eastern Greece
2013	86	11	20%	Area of Attica and North-eastern Greece
2014	15	0	0%	Area of Attica and North-eastern Greece

Table 1. Laboratory-confirmed case of West Nile Virus disease in Greece, 2010-2014.

This thesis reports several studies conducted to investigate possible implication of wild birds during the West Nile virus outbreak in Greece. Moreover, this thesis reports studies conducted to evaluate the application of novel technologies and tools such as Geographical Information Systems (GIS) and multiplex diagnostic techniques (microarrays) in wild birds surveillance programs, which have a potential to contribute in the future surveillance

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programmes designed to improve the monitoring the existing outbreaks and timely recognition of new outbreaks. .

In detail, the thesis is presented in the following chapters:

Chapter 1: Literature review about West Nile Virus, focusing on basic principles, replication mechanism, immune response and important genetic determinants of virulence.

Chapter 2: Investigation of wild avian samples for the presence of WNV RNA during the 2010 major outbreak of the disease in northern Greece.

Chapter 3: Extended investigation of the role of wild birds in the epidemiology of West Nile virus in Greece combining serological and molecular findings at the epicentre of the outbreak.

Chapter 4: Serological investigation of exposure of resident and migratory wild birds to the virus in the first years of the WNV outbreak in Greece and a first correlation of these results with human cases data.

Chapter 5: Use of wild bird surveillance, human case data and GIS spatial analysis for predicting spatial distributions of West Nile virus in Greece.

Chapter 6: Application of multiplex microarray technologies to wild avian tissue samples for screening West Nile virus and other avian viruses. Discussion on some technical aspects and considerations.

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CHAPTER 1

WEST NILE VIRUS: BASIC PRINCIPLES, REPLICATION MECHANISM, IMMUNE RESPONSE AND IMPORTANT GENETIC DETERMINANTS OF VIRULENCE

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1.1 Introduction

West Nile virus (WNV) was first isolated in Uganda (West Nile district) in 1937 from the blood of a native Ugandan woman [1] and until the end of the 20th century was considered a cause of viral encephalitis limited only in Africa and Asia. It became a global public health concern after the virus introduction in North America and especially New York in 1999 [2]. Before that, Romania had recorded the first large outbreak of West Nile neuroinvasive disease (WNND) in Europe in 1996, with 393 confirmed cases [3]. Since then, major outbreaks of WNV fever and encephalitis took place in regions throughout the world including America, Europe and Middle East, causing human and animal deaths. In the last decade, lineage 2 strains, considered of low virulence, have been introduced in Central and South Eastern Europe and were incriminated as causative agents of major human and animal disease outbreaks. A great number of WNV infections in humans occurred in 2010 and 2011 in Greece, with 363 laboratory confirmed cases and 44 deaths [4]. WNV lineage 2 strains were first detected from pools of *Culex* mosquitoes (strain Nea Santa-Greece-2010) [5] and a Eurasian magpie (strain magpie-Greece/10) [6] at the epicenter of the outbreak.

The unexpected high virulence of lineage 2 strains creates major concerns regarding the pathogenic potential of evolving and mutating WNV strains. The basic properties of WNV function will be presented focusing especially on the replication cycle, the pathogenicity mechanism as well as some important genetic determinants of virulence that have been recognized so far and can pose serious public health risks when present at various WNV strains.

1.2 Classification

West Nile Virus (WNV) is a member of the Flaviviridae family of single-stranded RNA viruses with linear non-segmented genomes. More than 58 members belong to the Flaviviridae family, whose name comes from the word “flavi”, Latin for “yellow”, because one of the most famous flaviviruses is the Yellow Fever Virus. Flaviviridae family is further divided in 3 genera: flaviviruses, pestiviruses and hepaciviruses. Pestivirus genus consists of 4 viral species that cause important animal diseases: Bovine Viral Diarrhoea Virus type 1 and 2, Border

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Disease Virus and Classical Swine Fever Virus. The only member of the Hepacivirus genus is Hepatitis C virus. The Flavivirus genus is the largest with at least 53 species divided into 12 serologically related groups. Of these, the Japanese Encephalitis Virus (JEV) group (8 species) is the one with the most human-associated disease viruses; Japanese Encephalitis Virus, St. Louis Encephalitis Virus, Murray Valley Encephalitis Virus and West Nile Virus are four members of the JEV group that have been associated with widespread human and animal disease outbreaks [7]. The International Committee of Taxonomy of Viruses can be consulted for the most accurate update regarding nomenclature and taxonomy of all viruses at the species level [8].

1.3 Structure and genome

The WNV genome is a positive single stranded RNA of approximately 11000 nucleotides surrounded by an icosahedral nucleocapsid which is contained in a lipid bi-layered envelope, of approximately 50 nm in diameter (Figure 1). The genome is transcribed as a single polyprotein that is cleaved by host and viral proteases into three structural (C, prM/M, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins [9] (Figure 2). Recent studies also reported that a larger NS1-like (NS1') viral protein, which is often detected during infection, is the possible result of ribosomal frameshifting [10].

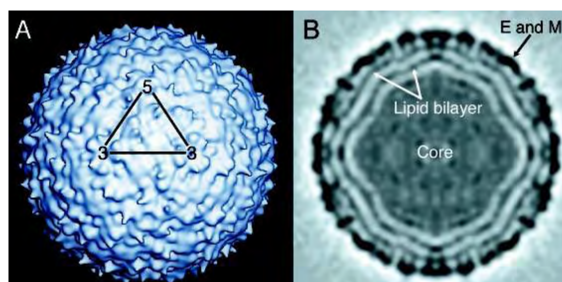

Figure 1. Structure of West Nile virus determined by cryo-EM. (A) A surface shaded view of the virion, one asymmetric unit of the icosahedron is indicated by the triangle. The 5-fold and 3-fold icosahedral symmetry axes are labelled. (B) A central cross section showing the concentric layers of density. Virion core, lipid bilayer and proteins E and M are indicated. Reprinted with permission from Science, 10 October 2003:248.DOI:10.1126/science.1089316.

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The viral capsid is approximately 30 nm in diameter and consists of C protein dimers, the basic component of nucleocapsids, with the RNA binding domains located at the C- and N- termini separated by a hydrophobic region [11]. The hydrophobic regions of the C dimers form an apolar surface which binds to the inner side of the viral lipid membrane [12]. In immature virions, the lipid bi-layered envelope that coats the nucleocapsid contains 180 molecules each of E and prM proteins organized into 60 asymmetric trimeric spikes consisting of prM-E heterodimers [13]. The transition from immature to mature virions starts with the release of the N-terminal prepeptide from the prM protein after cleavage by a furin-like protease in the trans-Golgi compartment of the infected cell [14].

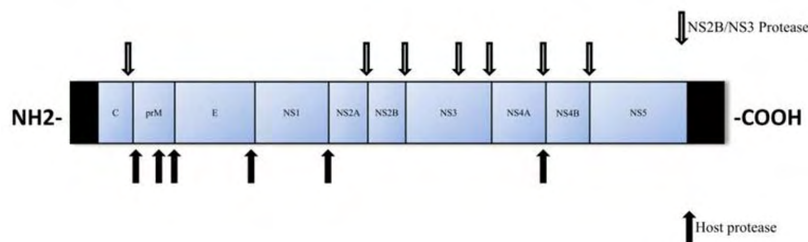

Figure 2. RNA genome of West Nile virus and site sites cleaved by host proteases and virus-encoded NS2B/NS3 protease.

Mature virions are characterized by the structural change, rotation and rearrangement of the 60 trimeric prM-E heterodimers to form 90 antiparallel homodimers with quasi-icosahedral symmetry that cover the lipid membrane [15, 16]. The E proteins are organized in 3 domains connected by flexible hinges [17]. Domain I (DI) is positioned at the central portion of the protein, linking together the other two domains. Domain II (DII) is a long domain containing a 13 residues long, glycine-rich, hydrophobic sequence that forms an internal fusion loop which is necessary for flaviviral fusion. Domain III (DIII) is an Ig-like fold that is thought to participate in interactions between virions and host factors associated with virus entry [18] (Figure 3).

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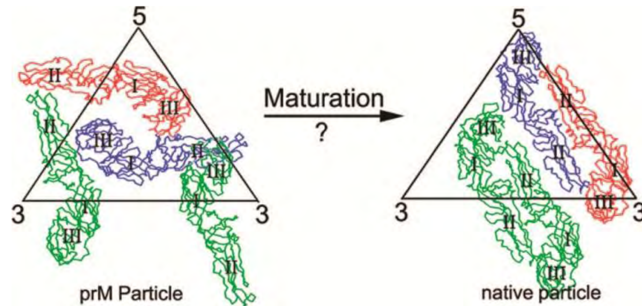

Figure 3. Diagram showing the structural rearrangement required for immature particles to become mature particles. The three independent E molecules per icosahedral asymmetric unit are coloured green, red, and blue. The three domains in each E molecule are labelled I, II, and III. Reprinted with permission from EMBO J. 22(11):2604-13.

The viral non-structural proteins are responsible for regulating viral mechanisms of transcription, translation and replication and attenuate host antiviral responses (Table 1). NS1 protein functions as a cofactor for viral RNA replication and is the only non-structural protein that is secreted in high levels (up to 50 μg/ml) in the serum of WNV infected patients and has been connected with severe disease [19]. Many theories have been proposed regarding the contribution of NS1 to the pathogenic mechanism of WNV: it has been proposed to elicit hazardous autoantibodies [20], to contribute to the formation of various immune complexes circulating in the host organism [21], antibodies against NS1 to cause endothelial cell damage [22], or to minimize immune response targeting of WNV by decreasing recognition of infected cells by the complement system [23].

NS2A is a hydrophobic, multifunctional membrane-associated protein which plays an important role in RNA replication [24] and viral particles assembly [25, 26]. NS2A is also the major suppressor of beta interferon (IFN-β) transcription, thus inhibiting interferon response, one of the first lines of defence of the host [27].

NS2B is a cofactor required for NS3 proteolytic activity. NS3 is a multifunctional protein, with two distinct functional domains. The protease comprises the N-terminal amino acid residues of NS3, while the carboxylated terminus contains a helicase, a nucleoside triphosphatase and a RNA triphosphatase [28 - 31].

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The NS3 trypsin-like serine protease is only active as a heterodimeric complex with its cofactor, NS2B. In the cytoplasm of infected host cells, this heterodimeric complex (NS2B-NS3pro) is responsible for post-translational cleavage of the viral polyprotein to release structural and non-structural viral proteins that are essential in viral replication mechanism and virions assembly. Cleavage takes place at the C-terminal side of two basic residues (e.g., RR, KK, and RK), a sequence motif that occurs at the junctions of NS2A/B, NS2B/3, NS3/4A, and NS4B/5. It also cleaves the viral polyprotein within the C-terminal region of protein C and protein NS4A as a necessary precursor to cleavage of prM and NS4B, respectively, by cell signalase in the lumen of the endoplasmic reticulum [28, 32]. The C-terminal of NS3 is characterized by the presence of motifs with homology to supergroup II RNA helicases, to a RNA-stimulated nucleoside triphosphatase (NTPase) and to a RNA triphosphatase (RTPase) [30, 33, 34]. The NTPase activity provides the chemical energy which is necessary to unwind RNA replication intermediates into forms that can be amplified by the NS5 RNA-dependent RNA polymerase [35, 36]. The RTPase dephosphorylates the 5' end of viral RNA, before cap addition by the N-terminal methyl transferase region of NS5 [37]. RNA helicases travel along RNA in a 3' to 5' direction fuelled by ATP hydrolysis; this movement opens secondary structures and displaces proteins bound to RNA [38]. Thus, together with the NS5 polymerase, with which NS3 is in tight association and interaction, the NS3hel plays an important role in flavivirus replication. However, a complete picture of the mechanism by which NS3hel associates with RNA template is not yet completely known.

NS4A, along with NS4B and NS2A, are the least known flavivirus proteins. The NS4A precise functional role has not been sufficiently characterized, although evidence suggests a role of "organizer" of the replication complex of flaviviruses. Its N-terminal is generated in the cytoplasm after cleavage by the NS2B-NS3 protease complex, whereas the C-terminal region (frequently designated 2K fragment) serves as a signal sequence for the translocation of the adjacent NS4B into the endoplasmic reticulum lumen. The 2K fragment is removed from the N terminus of NS4B by the host signalase, however a prior NS2B-NS3 protease complex activity at the NS4A/2K site is required [39]. Proteolytic removal of the 2K peptide also induces membrane alterations [40].

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Recently NS4A was proven to act as a cofactor for NS3 helicase allowing the helicase to sustain the unwinding rate of the viral RNA under conditions of ATP deficiency [41]. NS4B colocalizes with viral replication complexes and proved to dissociate NS3 from single-stranded RNA, thereby enabling it to bind to a new dsRNA duplex, consequently enhancing the helicase activity and modulating viral replication [42, 43]. In addition, NS4A and NS4B, along with NS2A, as has already been referred, and NS5 proteins appear to inhibit the interferon-α/β response of the host [44-46].

Finally, NS5 is the C-terminal protein of the viral polyprotein and is the largest and most conserved of flaviviruses proteins. The N-terminal region of NS5 contains an S-adenosyl methionine methyltransferase (MTase) domain, part of the viral RNA capping machinery. The cap is a unique structure found at the 5' end of viral and cellular eukaryotic mRNA, critical for both mRNA stability and binding to the ribosome during translation [47, 48]. The C-terminal region of NS5 contains a RNA-dependent RNA polymerase which is required for the synthesis of the viral RNA genome [49]. It was already mentioned that NS5 is in close interaction with NS3, constituting the major enzymatic components of the viral replication complex, which promotes efficient viral replication in close association with cellular host factors.

Non-structural Protein	Function
NS1	Cofactor for viral RNA replication, pathogenic mechanism in early infection (decrease complement recognition)
NS2A	Viral RNA replication and virions assembly, Major suppressor of IFN-β transcription
NS2B	Cofactor for NS3pro activity, interferons antagonist
NS3	Serine protease, RNA helicase, RTPase, NTPase
NS4A	"Organizer" of replication complex, inhibitor of interferon α/β host response
NS4B	Inhibitor of interferon α/β host response, enhancer of NS3hel activity
NS5	Methyltransferase, RNA-dependent RNA polymerase, interferon antagonist

Table 1. Functions of West Nile virus (WNV) non-structural proteins.

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1.4 Replication cycle

WNV has the ability to replicate in various types of cell cultures from a wide variety of species (mammal, avian, amphibian and insect) (Figure 4). The first step in the infectious cell entry involves the binding of E protein to a cellular molecule-receptor [50]. Several cell molecules have been proven to function as co-receptors for in-vitro virion attachment: WNV interacts with DC-SIGN and DC-SIGN-R in dendritic cells [51]. It has been documented to attach to the integrin αvb3, through DIII RGD/RGE sequence, which is an integrin recognition motif [52]. However a recent study showed that WNV entry does not require integrin αvb3 in certain cell types suggesting that receptor molecule usage is strain-specific and/or cell type-dependent [53].Rab 5 GTPase was found to be a requirement for WNV and Dengue Virus cellular entrance [54]. Laminin binding protein is also a putative receptor for the WNV, with proved high specificity and efficiency between LBP and DII of E protein [55, 56]. Many other attachment factors have been identified for flaviviruses, including CD14 [57], GRP78/BiP [58], 37-kDa/67-kDa laminin binding protein [58], heat-shock proteins 90 and 70 [59], and even negatively charged lycoaminoglycans, such as heparan sulphate, which are expressed in various cell types, though, for the latter, recent studies did not reveal specific binding of WNV with heparan sulphate [60].

After the viral attachment via the cellular receptors, WNV enters the cell through clathrin- mediated endocytosis [61]. It is characteristic that it was possible to inhibit WNV infection by treating cells with chemical inhibitors like chloropromazine [62] that prevent the formation of clathrin-coated pits, or by expressing negative mutants of Eps15 in cells. Eps15 is a protein involved in clathrin-coated pit formation [63]. The endosome environment is characterized by acidic PH, which triggers conformational changes of the E glycoprotein. The first step involves the disruption of the E protein rafts and dissociation of the E homodimers to monomers. An outward projection of DII takes place, and the fusion loop of DII is exposed to the target membrane. The E proteins insert their fusion loops into the outer leaflet of the cell membrane. Three E monomers interact with one another via their fusion loops to form an unstable trimer which is stabilized through additional interactions between the DI domains of the three

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E proteins [50, 64]. Next, DIII is believed to fold back against the trimer to form a hairpin-like configuration. The energy released by these conformational changes induces the formation of a hemifusion intermediate, in which the monolayers of the interacting membranes are merged. Finally, a fusion pore is formed and after enlargement of the pore, the nucleocapsid is released into the host cell. The viral RNA is released by the nucleocapsid with a yet unknown mechanism and is translated. The produced polyprotein is cleaved at multiple sites by the NS3 serine protease and the host signal peptidase within the lumen of the endoplasmic reticulum. At the same time, the viral RNA-dependent RNA polymerase copies complementary negative polarity (–) strands from the positive polarity genomic (+) RNA template, and these negative strands serve as templates for the synthesis of new positive viral RNAs. Studies showed that RNA replication can continue without protein synthesis, and that from a (+) strand RNA only one (-) strand RNA can be synthesized at a time, while from a (-) strand RNA multiple (+) strand RNAs can be simultaneously copied [65,66]. However virion assembly cannot take place if sufficient protein synthesis has not been performed: Each virion contains 180 copies each of E and prM structure proteins and only one genomic copy.

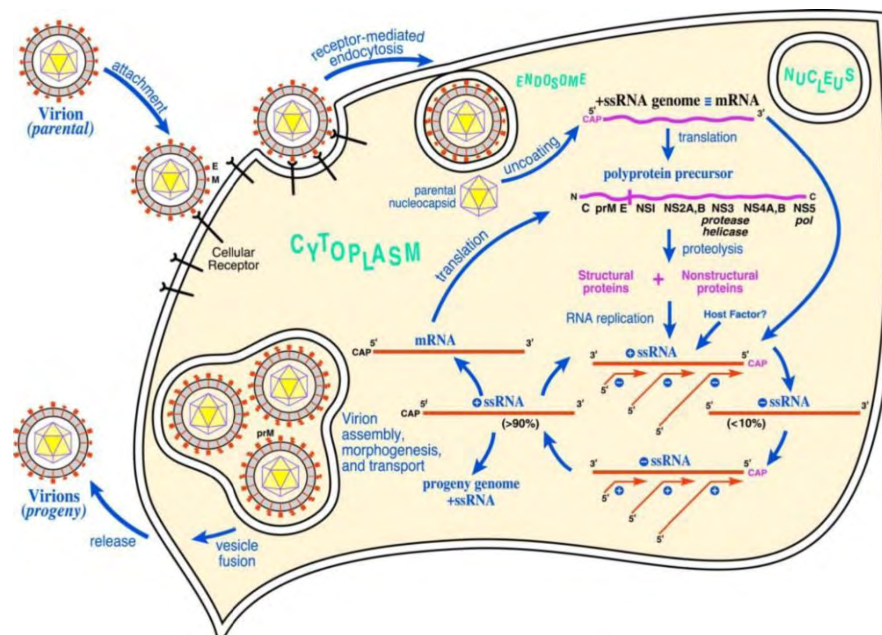

Figure 4. West Nile virus replication cycle. The virion is attached to the cellular membrane of thee host cell via the cellular receptors, and the envelope fuses

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with the membrane. The viral RNA is released by the nucleocapsid with a yet unknown mechanism and serves as mRNA for translation of all viral proteins and as template during RNA replication. Virion assembly and release of them to the extracellular milieu complete the replication cycle. Reprinted with permission from PNAS 2002, vol. 99 no. 18 11555-11557. Copyright 2002 National Academy of Sciences, U.S.A.

During West Nile virion assembly, C proteins bind to the newly replicated RNA and wrap around it to form an icosahedral shell. This nucleocapsid will be enveloped by cellular membrane derived from the endoplasmic reticulum and will bud into the lumen as immature virions on which E and prM proteins form 60 heterotrimeric spikes. Immature virions are then transported to the mildly acidic compartments of the trans-Golgi network triggering a rearrangement of E proteins on the immature virion; the lower pH induces a structural transition such that E proteins form 90 antiparallel homodimers on the surface of the virion [67] (Figure 4). Under acidic conditions, prM remains associated with the virion and protrudes from the surface of an otherwise smooth virus particle. This pH-dependent conformational change increases the susceptibility of prM for a furin-like serine protease [68].The pr peptide dissociates from the particle upon release of the virion to the extracellular milieu by exocytosis, which starts 10-12 h after cell infection. However, this furin processing of prM is rather inefficient and many virions still contain prM proteins even after their release to the extracellular milieu, which will reorganize back to prM/E heterodimers.

This inefficient and incomplete maturation leads to the secretion of a mixture of mature, immature and partially mature particles from flavivirus-infected cells. A high number of prM-containing particles have been described for WNV. Until recently, fully immature virions were considered to be unable to cause infection as they cannot undergo the structural rearrangements required for membrane fusion [69]. However, newer studies proved that even fully immature virions of flaviviruses can cause infection by antibodies [70, 71]. Regarding partially immature virions, multiple studies have shown that they can also be infectious [17, 72]. It seems that the mature part of these virions is responsible for cell binding and entry after which the further processing of remaining prM may take place inside the cell. Further studies are needed to estimate the “cut-off”

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regarding the number of prM proteins on viral surface that allow the viral particle to be infectious.

1.5 Epidemiology

Avian species are considered the primary hosts of West Nile virus, and in an endemic region, virus is maintained in an enzootic cycle between mosquitoes and birds [73]. Birds from more than 300 avian species have been reported dead from West Nile virus [74]. Disease can also be caused in humans and other mammals, particularly horses, considered as alternative hosts of WNV; main route of infection is through the bite of infected mosquitoes. However, the virus can also spread between individuals by blood transfusion and organ transplantation and few reports have also proposed the transmission from mother to new-born via the intrauterine route or via breast-feeding [75-77]. Most human infections remain asymptomatic, West Nile fever (a mild flu like fever) develops in approximately 20 to 30% of infected persons and West Nile neuroinvasive disease in <1% [78], characterized by encephalitis, meningitis, acute flaccid paralysis and even long-term neurological sequeale [79]. Nonetheless, horses and humans develop viremia levels of low magnitude (<10^5 PFU/ml) and short duration insufficient to infect mosquitoes and thus do not serve as amplifying hosts for WNV in nature [80]. On the contrary, various avian species, both migratory and sedentary, develop viremia levels sufficient to infect most feeding mosquitoes [81]. Hence, WNV is maintained in an enzootic cycle with wild and domestic birds being the main amplifying hosts and ornithophilic mosquitoes, especially of the Culex species, the main vectors. Moreover, local movements of resident birds and long- range travel of migratory birds may both contribute to the spread of WNV [82, 83]. Various studies have provided indirect evidence that WNV is transported by migratory birds, especially via their migration routes from breeding areas of Europe to wintering areas in Africa [84-87].

WNV strains are grouped into at least 7 genetic lineages [88] (Figure 5). Lineage 1 is the most widespread, containing isolates found in Europe, North America, Asia, Africa and Australia. This linage is further divided into at least two different clades: WNV-1a is found mainly in Africa, Europe, North America and Asia and is further divided in six evolution clusters [89]. WNV 1-b contains

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the Australian Kunjin virus. A third clade containing Indian isolates is now classified as Lineage 5 [90]. Lineage 2 strains are mainly distributed in Sub-Saharan Africa and Madagascar, but in the last decade they have been introduced in Europe. Lineage 3 contains a strain circulating in certain Culex and Aedes species mosquitoes in southern Moravia, Czech Republic, namely “Rabensburg virus”, not known to be pathogenic to mammals [91]. Lineage 4 is represented by a strain isolated from Dermacentor marginatus ticks from the Caucasus [92]. A re-classification of Sarawak Kunjin virus as lineage 6 has been proposed as this strain is different to other Kunjin viruses. The African Koutango virus is closely related to WNV and a seventh lineage has been proposed for this strain. An eighth lineage has been proposed for WNV strains detected in Culex pipiens mosquitoes captured in Spain in 2006, which could not be assigned to previously described lineages of WNV [93].

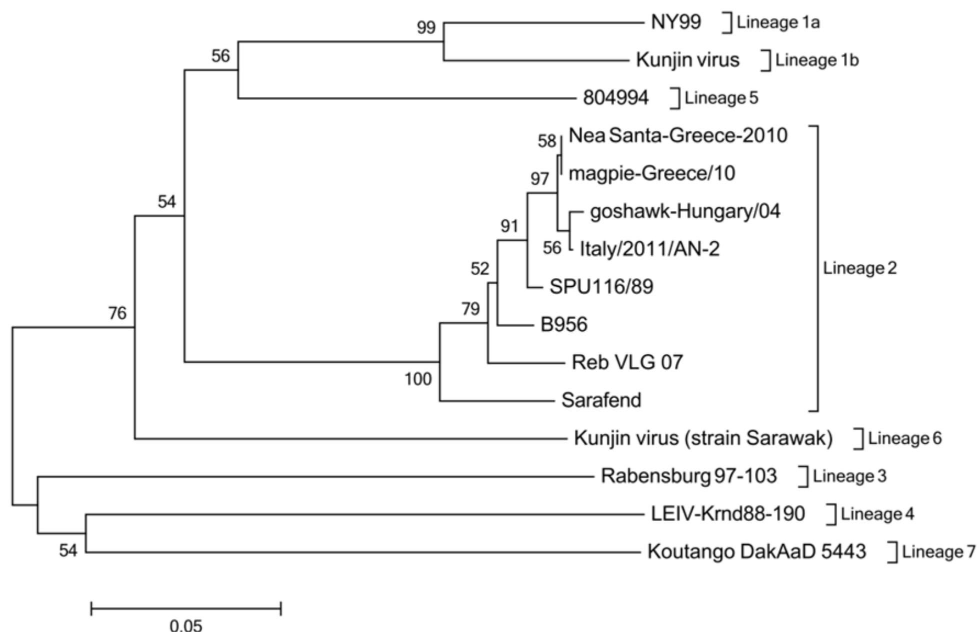

Figure 5. Phylogenetic tree of a 236-nt NS5 genomic region. Phylogenetic analysis based on a 236-nt NS5 genomic region of 15 West Nile virus strains, representatives of all recognized lineages, focusing on Lineage 2 strains circulating in South Eastern Europe. Analysis was performed using MEGA version 5. GenBank accession numbers and geographic origins of strains used in this analysis are: NY99 (AF202541, USA); Kunjin virus (D00246, Australia); 804994 (DQ256376, India); Nea Santa-Greece-2010 (HQ537483, Greece);

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magpie-Greece/10 (JQ954395, Greece); goshawk-Hungary/04 (DQ116961, Hungary); Italy/2011/AN-2 (JN858070, Italy); SPU116/89 (EF429197, South Africa); B956 (AY532665, Uganda); Reb VLG 07 (FJ425721, Russia); Sarafend (AY688948, Israel); Kunjin virus/strain Sarawak (L49311, Malaysia); Rabensburg 97-103 (AY765264, Czech Republic); LEIV-Krnd88-190 (AY277251, Russia); Koutango DakAaD 5443 (L48980, Senegal). Neighbour-joining tree was constructed from a difference matrix employing the Kimura 2-parameter correction. One thousand bootstrap pseudo- replicates were used to test the branching (shown as percentages, with a cut-off value of 50%).

Lineage 2 was considered to be endemic in Sub-Saharan Africa and Madagascar, however, since 2004 strains have been observed in Hungary from birds of prey [94] and in 2007 in Russia from mosquito pools during a disease outbreak with 67 human cases [95]. In 2010 it caused outbreaks in Romania [96] and Greece [4] and in 2011 it was detected for the first time in Italy [97, 98]. The Greek and Italian strains showed the highest homology to Hungarian and South African strains, differing from the Russian lineage 2 strains detected in 2007. However, in Italy no major human disease outbreak occurred; only one human case was reported with mild clinical expression [97]. Genetic analysis of the Italian strains revealed the presence of histidine at 249 aa position of NS3, just like the Hungarian strains, in contrast to the Greek strains that contained proline at that position, the presence of which has been already implicated with high pathogenicity of lineage 1 strains [99].

1.6 Pathogenesis

Most of our knowledge regarding WNV dissemination and pathogenesis derives from the study in rodent models. After an infected mosquito bite, WNV infects keratinocytes and Langerhans cells [100,101] which migrate to lymph nodes resulting in a primary viremia [102]. Then the virus spreads to peripheral visceral organs like kidney and spleen where a new replication stage occurs, in epithelium cells and macrophages respectively [103]. Depending on the level of viremia, the peak of which comes at day 3 p.i. in mice, the virus may cross the blood-brain barrier (BBB) and enter the central nervous system (CNS), causing meningo-encephalitis. Various ways have been proposed for WNV entry to CNS; TNF-a mediated change in endothelial cell permeability have

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been proposed to facilitate CNS entry [104], as well as infection of olfactory neurons and spread to the olfactory bulb [105]. Other ways involve direct axonal retrograde transport from infected peripheral neurons [106] or transport of the virus by infected immune cells trafficking to the CNS [107]. WNV infects neurons in various parts of the CNS causing loss of architecture, degeneration and cell death. In a later stage mononuclear cells infiltrate the infected regions although it is not really clear if they help stop infection or contribute to pathogenesis destroying infected cells and releasing cytokines [108]. Infection and injury of brain stem, hippocampal and spinal cord is observed in both humans and rodents that succumb to the disease. Persistence of WNV in mice was found to be tissue dependent. Infectious virus could persist as long as 4 months p.i., especially in mice that did not exhibit disease during acute infection and especially in the skin and spinal cord [109]. This persistence may also occur in humans after mild febrile illness or subclinical infections; 3% of WNV-positive blood donors were found to have detectable WNV RNA in blood between 40 and 104 days after their index donation [110].

In wild birds, less is known regarding pathogenesis of WNV. The virus has been detected by histology and RT-PCR in various tissues e.g. brain, liver, lungs, heart, spleen and kidneys of various avian species e.g. crows, blue jays, goshawks, magpies [111, 112, 94, 6]. Various avian species were found to be viremic for 6 days post inoculation and viremic titres high enough to transmit the virus to mosquitoes via their bites [113]. In wild birds, infectious WNV was detected for as long as 6 weeks in tissues [114,115]. However it is important to clarify that immune response, virulence and viral persistence is to a great degree species dependent, with great variations among various avian species in different geographical areas, as well as strain dependent, implicating various genetic determinants of virulence.

1.7 Immune response

Immune response of animals and humans to WNV infection is divided to innate and adaptive.

Innate response includes interferons, complement and innate cellular immunity

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Interferons type I (IFN-α and IFN-β), type II (IFN-γ), and type III (IFN- λ) IFNs play an essential protective role limiting infection of many viruses. IFN-α/β is produced by most of the cells following viral infection and induces an antiviral state to the cell, "activating" the relevant genes. It also creates a linkage between innate and adaptive immune responses by various mechanisms e.g. activation of B and T cells or dendritic-cell maturation [116, 117, 118]. IFN-γ is produced by γδ T cells, CD8+ T cells, and natural killer cells and limits early viral dissemination to the CNS through several mechanisms [119, 120]. WNV has evolved various countermeasures, at least 6 different mechanisms, against interferons function [121]. Hence, IFN administration cannot be considered of significant therapeutic importance for WNV disease control [122].

Several nucleic acid sensors e.g. TLR3, cytoplasmic dsRNA, RIG-I and MDA5 bind to viral RNA and activate transcription factors like IRF3 and IRF7 as well as IFN-stimulated genes [123-126].

Complement is a system of proteins in serum and molecules on cell surface that recognize pathogens and induce pathogen clearance. Three pathways exist for complement activation the classical, the lectin and alternative pathways, which are initiated by binding of C1q, mannan-binding lectins or hydrolysis of C3 respectively. All three pathways have been found to be important for controlling WNV lethal infections [127- 129].

There is data suggesting that macrophages and dendritic cells may directly inhibit WNV. Macrophages can control infection through cytokine and chemokine secretion, enhanced antigen presentation and direct viral clearance [130]. γδ T cells also limit WNV infection in an early stage [131].

Adaptive response includes humoral and cellular response

Humoral immunity plays a vital role in protection from WNV infection. Experimental studies demonstrated complete lethality of B-cell-deficient and IgM-/- mice infected with WNV, whereas they were protected by transfer of immune sera [132,133]. IgM titres at day 4 p.i. could predict the disease outcome at prospective experiments. IgG can also protect from infection, however, in primary infection their role is less vital: Being produced after days 6-8, the disease outcome has been determined, since both viral shedding to

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CNS and clearance from tissues have already occurred [132, 134]. The vast majority of neutralizing antibodies are directed against all three domains of E protein. However the most potent neutralizing antibodies are directed on DIII possibly inhibiting viral fusion at post-attachment stage [135, 136]. In humans, antibodies against prM have also been recognized but with limited neutralizing activity [70, 137, 138]. Antibody neutralization is a procedure where multiple antibodies, above an estimated threshold "manage" to neutralize the virion's activity and render it non-infectious. This threshold was estimated to be 30 antibodies per virion for a highly accessible epitope of DIII of E protein [139-141]. It is important, however, to understand the following aspects: The level of neutralizing antibodies does not always correlate with protection against WNV. WNV have 180 E proteins on their surface. Steric phenomena because of the dense icosahedral arrangements of these proteins do not allow the equivalent display of all the epitopes. There are also many structurally distinct epitopes, not easily accessible to certain neutralizing antibodies. It is characteristic that studies showed a reduction of the neutralizing ability of antibodies correlated to the maturation state of WNV: Maturation reduces the accessibility of some of the epitopes on the virion [17]. Thus, these antibodies cannot efficiently neutralize the virus even if at high levels of concentration. This can lead to completely different result: Antibody dependent enhancement (ADE) of infection is possible in cells bearing activating Fc-γ receptors [141, 142] and thus a mild infection with sufficient levels of antibodies can become even life-threatening due to the inability of the antibodies to neutralize the virions.

Antibodies against NS1, a protein secreted in the serum of patients during acute phase of disease and expressed on the surface of infected cells considered to be a cofactor in virus replication, have been found to be non-neutralizing but protecting through both Fc-γ receptor-dependent and independent mechanisms [143].

T lymphocytes (part of cellular response mechanism) have been demonstrated to be vital for the protection against WNV infection. Recognizing an infected cell through the viral antigen fragments associated with MHC class I molecules on the infected cells' surface, cytotoxic (CD8+) T cells secrete cytokines and lyse the cells directly (perforin, granzymes A and B) or indirectly via Fas-Fas

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ligand interactions [144, 145]. Studies showed that for the protection against lineage I, perforin played the most important role and, in contrast, lineage II strain Sarafend was controlled more efficiently by granzymes [146, 147]. CD4+ T cells contribute through multiple mechanisms, and preliminary data suggest that CD4+ T cells restrict pathogenesis in vivo [148]. Except IFN–α/β, T-cell immune response is extremely essential regarding the control of WNV in the CNS, their presence being correlated with virus clearance [146, 149, 150]. WNV infection induces the secretion of the chemokine CXCL10 from neurons, recruiting effector CD8+ T cells via the chemokine receptor CXCR3 [151].Expression of chemokine receptor CCR5 and its ligand CCL5 is up-regulated by WNV and is associated with CNS infiltration of CD4+ and CD8+ T cells, NK1.1+ and macrophages expressing the receptor [152].

All the above data provide solid evidence that a combination of various aspects of both innate and adaptive immune response cooperate to control WNV infection in the periphery and CNS.

1.8 Genetic determinants of virulence

Various studies especially in the last decade have recognized a variety of genetic determinants of virulence for West Nile virus strains. Specific mutations have been found to attenuate or strengthen virus pathogenicity via various mechanisms. Those that have been found to be the most important will be reported here, focusing on the ones that seem to have major impact on the replication mechanisms of WNV.

Mutations at the Envelope protein at residues 154 to 156, which abolished the N-linked glycosylation motif (N-Y-S/T) was proved to attenuate virus pathogenicity in mouse models [153]; these mutations seem to alter the protein such that it cannot be recognized by oligosaccharyl-transferase, thus glycan loss is caused [154]. This glycosylation motif has been recognized to various flaviviruses and spatially is located in close proximity to the centre of the fusion peptide of DII of E protein, and thus is considered to increase the stability of the protein to a fusion-active form even at high temperatures [155, 156]. This proved to be really important for the multiplication of the virus to avian cell and animal models: results showed that E glycosylated WNV variants multiplied

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more efficiently to avian cell cultures and at high temperatures, causing at the same time high viremic titres and pathogenicity to chicks [157]. Most of the Lineage I virulent strains as well as recent virulent Lineage II strains associated with the Greek outbreak carry the N-glycosylation site, suggesting it a prerequisite for the efficient circulation and amplification of the virus in a mosquito-avian transmission cycle [158]. Of course it is possible that E glycosylation affects other aspects of the WNV replication cycle as well such as target cell tropism, virion assembly and release etc.

We have already referred to the NS4B protein proven dual role of involvement to virus replication mechanism (enhances helicase activity) and evasion of host innate immune defence (inhibits IFN α/β response). Studies proved that substitution of cysteine (an amino acid which is often critical for the proper function of a protein) with serine at position 102 of NS4B, (Cys102Ser) leads to sensitivity to high temperatures as well as attenuation of the neuroinvasive and neurovirulent phenotypes in mice [159]. It was determined previously that the first 125 amino acids of the N-terminal of NS4B protein of flaviviruses are sufficient for the inhibition of IFN-α/β signalling [160]. Hence, this mutation which is located in this region of WNV may attenuate the viral ability to inhibit IFN signalling. Attenuation of the viral pathogenicity, characterized by lower viremia levels and no lethality to mice, was caused by a P38G mutation in the NS4B protein [161]; this was proven to be related to an induce of higher innate and adaptive immune response in mice, with higher type I IFNs and IL-1β levels and stronger memory and effector T cells responses. An adaptive mutation (E249G) in the NS4B gene resulted in reduced in-cell viral RNA synthesis, probably affecting the involvement of NS4B to the virus replication mechanism [162].

NS2A protein, as already stated, plays important role in RNA replication and viral particles assembly, and is also the major suppressor of IFN-β transcription. It was found that an A30P mutation of a Kunjin subtype WNV strain resulted in a reduced ability of the virus to inhibit IFN response, leading to increased levels of IFNs synthesis [27]. However this mutation implemented in North American Lineage 1 strains did not cause significant changes to phenotype indicating that in many cases the effect of mutations under study can be strain- specific. D73H

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and M108K were mutations found to be related to poor replication and non-mortality to mice [163].

NS3 protein includes the serine protease at the N-terminal and the RNA helicase, an NTPase and an RTPase at the C-terminal. The introduction of a T249P in North American Linage 1 strain was found to be sufficient to generate a phenotype virulent to American crows [99]. A H249P mutation is considered to be the main cause of increased virulence of Lineage 2 strain that caused the major WNV disease outbreak in 2010-2011, in Greece. Only the Greek sequences, detected in mosquito pools, corvids and chickens [5, 164] contains proline at this locus, while all other Lineage 2 strains contain histidine. The exact mechanism through which this mutation increases the pathogenicity of WNV is unknown, believed though to be related to increased replication rate caused by an enhancement in RNA helicase function; hence, the virus may surpass bird viremia thresholds required for infection of many mosquito species vectors (> 10^5 PFU/ml). However, recent studies on European Lineage 1 strains Morocco/2003 and Spain/2007 proved that the first was more pathogenic in a mouse model than the second; Morocco/2003 contains a T and Spain/2007 a P at 249 aa position. Hence, a proline residue in position 249 of the NS3 position is not sufficient to enhance virulence, at least in certain cases [165, 166]. Another study detected a potential role of a S365G mutation to enhance viral replication, by lowering the requirement of ATP for ATPase activity, thus allowing the RNA helicase to sustain the unwinding rate of viral RNA under conditions of ATP deficiency [167].

The function of the hydrophobic 2K peptide that spans the ER membrane between NS4A and NS4B remains largely unknown. It is believed that it acts as signal sequence for the translocation of NS4B into the ER lumen. It is removed from the N-terminus of NS4B by a host ER signalase. 2K-V9M mutant virus generates higher viral titres in Oas1b-expressing cells than the wild type virus. The exact mechanism by which the 2K-V9M substitution enables WNV resistance to antiviral action of Oas1bis unknown [167].

Theoretically, substitutions of hydrophobic to hydrophilic amino acids and vice versa as well as substitutions of glycine, proline and cysteine residues are considered to have a potential effect on the secondary structure of proteins. A

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study performed on Lineage 2 strains of low and high virulence recognized this kind of substitutions at NS3 (S160A and R298G), NS4A (A79T) and NS5 protein (T614P, M625R, M626R) that were present at high virulent strains [168].

1.9 Conclusions

West Nile virus is considered a serious public health threat, especially for high risk groups (very young and elderly, immunocompromised). Currently there has not been established any antiviral treatment to WNV infections; only supportive care may be administered. Vaccine development is still at an early stage for humans. Hence, preventive measures rely still on reduction of mosquito populations and minimizing vector-host contact. Various diagnostic techniques have been developed the last decades, both molecular and serological, trying to minimize the difficulties arisen from other cross-reactive closely related flaviviruses. Data presented here prove the complexity of the host-virus interaction: Specific host-pathogen- vector interface, cellular tropism, viral structure diversity regarding maturation, immune system recognition and response, genetic diversity are all factors characterized by great variation rendering WNV control extremely difficult. Continuous studies are being demanded to understand the extent of this complexity to further elucidate biological relationships among host, vector and virus that will lead to improved disease control. As more is learned about the biological characteristics of WNV infection, one continuing objective will be to relate this knowledge to the clinical features of disease. An important viral-host determinant is virus attachment, mediated by cellular receptor and allowing subsequent infection. Host defensive behaviours that could affect virus acquisition and transmission should be also further studied. This may help in the design and implementation of more efficient and cost-effective control strategies since introduction of WN virus is an ongoing risk and reality. The ultimate challenge will be to apply the knowledge gained in understanding viral replication and unravelling the complexity leading to pathogenesis in order to prevent and control West Nile virus and its severe manifestations.

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CHAPTER 2

MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF WEST NILE VIRUS LINEAGE 2 IN SEDENTARY WILD BIRDS (EURASIAN MAGPIE), GREECE, 2010

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Summary

A West Nile virus (WNV) lineage 2 strain was molecularly identified and characterised in a Eurasian magpie hunted in Greece in 2010, during a WNV outbreak in humans. Phylogenetic analysis revealed the highest sequence similarity (>99%) with other WNV lineage 2 strains derived from birds of prey in Austria and Hungary (2004–2009). This first molecular detection of WNV in sedentary wild birds in Greece, which are possible reservoirs of the virus, is a public health concern.

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2.1 Introduction

West Nile virus (WNV) is a mosquito-transmissible Flavivirus with zoonotic potential. The virus has been present in Europe for decades; however, only recently were strains of lineage 2 (L2) identified outside of Africa: in 2004 and 2005 in goshawks in Hungary, in 2007 in Volgograd, Russia, and in 2008 and 2009 in goshawks and a falcon in Austria [1-3]. From early July through October 2010, 261 laboratory-confirmed cases of WNV infection in humans were reported in northern Greece as part of an outbreak. Of these, 191 patients presented with neuro-invasive symptoms, and 34 deaths were reported [4]. Most cases were observed in central Macedonia, in areas located between four major rivers (Axios, Loudias, Aliakmon and Gallikos) which converge into a common delta, a well-known resting and breeding ground for migratory birds.

2.2 Methods and Results

The objective of our study was to detect possible infection of wild birds with WNV during the outbreak in Greece, and to molecularly characterize and define the WNV strain geographical origin in positive samples.

Our first focus was on members of the Corvidae family. Many corvid species are sedentary and territorial, having a wide daily dispersal range of up to 20 km, social, roosting in large colonies and abundant in both wetlands and urban areas [5]. Hence, introduction of the virus in an area (i.e. via migratory birds) may result in its transmission, circulation and maintenance in local corvid populations. Samples from hunter-harvested corvids (Eurasian magpies and carrion crows, hunted species according to Greek law) were collected during the hunting season (from 20 August until 28 February the following year) of 2009/10 and of 2010/11. Sampling was carried out in the municipalities of Thermi and Axios (prefecture of Thessaloniki, central Macedonia, Greece) by members of the Hunting Federation of Macedonia and Thrace, locating corvid roosting sites in nearby wetlands. Hunters were briefed on signs of encephalitis in birds, and were instructed to report any such observations. No findings of birds with signs of encephalitis or dead birds were reported from any of the hunters.

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Of 96 corvids collected, 36 were tested, including 28 Eurasian magpies (Pica pica) and eight carrion crows (Corvus corone). A pool of selected tissues (kidney, heart, liver) was created from each bird. RNA was extracted from each pool, which constituted a single sample, using the PureLink RNA Mini Kit (Invitrogen). An -RT-PCR specific for Japanese encephalitis virus complex was performed for all extracts resulting in a 1,084-bp amplification product covering part of the non-structural protein 5 (NS5) gene, as described earlier [6]. A band of expected size was obtained from one PCR product derived from a magpie harvested near the village of Trilofos (40°28'25.57"N, 22°58'28.62"E) in September 2010 (Figure 1). A serum sample from the magpie in question was tested for the presence of WNV IgG antibodies by indirect immunofluorescence test using a commercial kit (EUROIMMUN) [7]; the serum sample was positive at a dilution of 1/30.

The positive PCR product was purified using the PureLink PCR Purification Kit (Invitrogen) and was bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems), followed by fragment separation with a 3,730xl DNA Analyser (Applied Biosystems).

Phylogenetic analysis was conducted using MEGA 3.1 [9]. Nucleotide sequences from other WNV strains were retrieved from Genbank (NCBI). Phylogenetic analysis of 797 nucleotide-long partial NS5 sequences was performed. A neighbour-joining phylogenetic tree using Kimura-2 parameter distance matrix was inferred from 26 WNV strain sequences (including that derived from the magpie in our study) and two sequences of the Japanese Encephalitis virus complex as outgroups (Figure 2). Node support was assessed with 1,000 bootstrap pseudo-replicates.

The WNV sequence derived from the Greek magpie clustered with WNV L2 strain sequences and presented highest (99.9%) sequence similarity to L2 strain sequences derived from birds of prey in Austria obtained in 2008 and 2009 [2]. A 99.6% similarity was also observed with the corresponding region of an L2 strain derived from a dead goshawk in Hungary in 2004 [1]. No amino acid changes were observed in the genomic region of the magpie derived WNV strain compared to Austrian and Hungarian strains. According to our analysis, all these strains as well as two strains from South Africa belong to the same

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sub-cluster. A lower sequence similarity (96.8%) was observed with a WNV L2 strain isolated during an outbreak in Russia in 2007. The Russian strain sequence groups with other African strains (including other South African strains) in a separate sub-cluster, suggesting a different reintroduction of WNV L2 in Europe [3]. The sequence from the Greek magpie isolate was deposited in GenBank under accession no. JF719073.

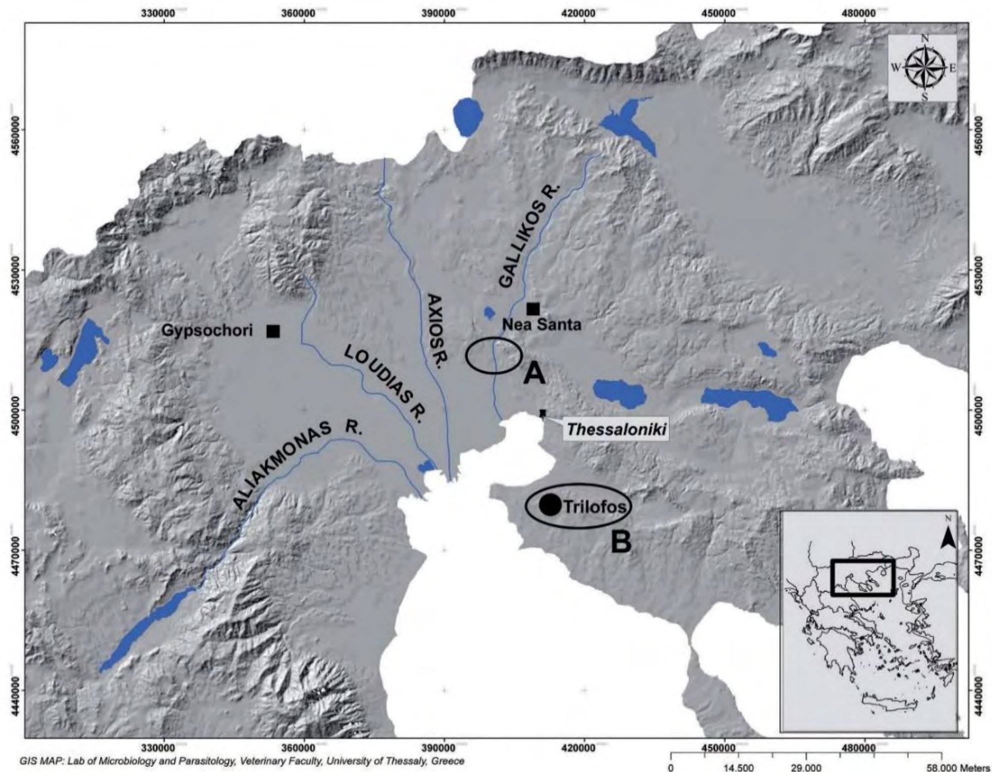

Figure 1. Bird sampling area for West Nile Virus, Central Macedonia, Northern Greece, 2009–2011. WNV: West Nile Virus.

The study area corresponds to the areas where most human cases occurred during the WNV outbreak. Black square boxes indicate where WNV was detected in mosquitoes [8]. A and B indicate areas, where tested corvids were harvested. The black circle indicates where the WNV-positive Eurasian magpie was hunted.

2.3 Discussion

From early July through October 2010, a WNV outbreak in humans occurred in northern Greece, as confirmed by serologic evidence. To date, no WNV

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genomic sequences are available from the human cases during this outbreak. A WNV strain sequence derived from a magpie hunted during the outbreak of the human disease was found in this study. The sequence has highest sequence similarity to L2 strain sequences from birds of prey in Austria obtained in 2008 and 2009. WNV RNA fragments, though limited in size, (146 nt NS5 genomic region) with 100% sequence similarity to Hungarian and Austrian L2 strains, were also detected in two pools of mosquitoes caught during the time of the Greek outbreak and in the same area [8]. The mosquito WNV sequence was not included in our analysis because it did not overlap with the magpie WNV sequence. However, the similarity of both to the Austrian L2 strain sequences suggests that the same WNV strain is implicated in the magpie and mosquito infections and associated with the human outbreak. The evidence may implicate this corvid species in a local virus maintenance and generates concerns about possible overwintering and expansion of the virus in neighbouring areas. To test this hypothesis, research must be extended in non-epidemic periods, by performing molecular and serologic surveillance in wild birds and focusing efforts on the isolation of infectious WNV from avian samples.

Phylogenetic analysis of our strain revealed a high sequence similarity with Austrian and Hungarian WNV strains detected in previous years in birds of prey (2004–2009). According to these findings, it can be hypothesized that the virus expanded from northern Europe southwards. The area of the recent outbreak is a well-known resting and breeding ground for migratory birds passing on the way from nesting grounds in Europe to wintering areas in Africa. Re-introduction of the virus in the future by birds migrating along the south-eastern migration route that leads from Europe and western Asia to Africa should also be considered possible and needs further investigation.

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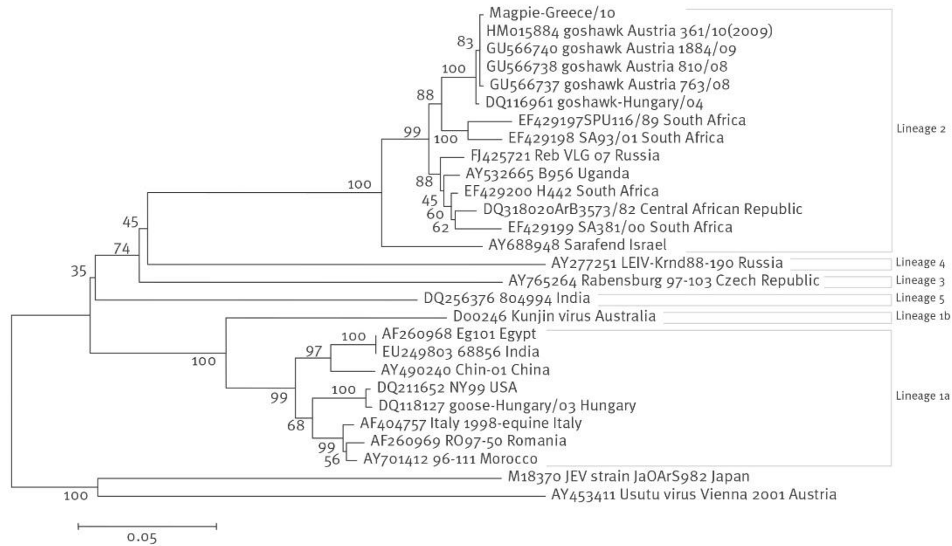

Figure 2. Phylogenetic tree of West Nile Virus strains based on nt sequences of the NS5 genomic region.

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CHAPTER 3

SEROLOGICAL AND MOLECULAR INVESTIGATION INTO THE ROLE OF WILD BIRDS IN THE EPIDEMIOLOGY OF WEST NILE VIRUS IN GREECE

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Summary

Background

A West Nile virus (WNV) disease outbreak occurred in 2010 in northern Greece with a total of 262 laboratory-confirmed human cases and 35 deaths. A serological and molecular surveillance was conducted on samples of hunter-harvested wild birds prior to and during the outbreak.

Findings

Serum and tissue samples from 295 resident and migratory wild birds, hunter-harvested during the 2009–2010 and 2010–2011 hunting seasons at the epicenter of the outbreak in northern Greece, were tested for the presence of WNV-specific antibodies by immunofluorescence assay and virus neutralization test. WNV neutralizing antibodies were detected in 53 avian samples. Fourteen positive sera were obtained from birds hunter-harvested up to 8 months prior to the human outbreak. Specific genetic determinants of virulence (His249Pro NS3 mutation, E-glycosylation motif) were recognized in a WNV lineage 2 strain isolated from a hunter-harvested Eurasian magpie and a nucleotide mismatch was revealed between this strain and a mosquito WNV strain isolated one month earlier in the same area.

Conclusions

This is the first report regarding exposure of wild birds to WNV prior to the 2010 outbreak, in Greece. Results provide evidence of the implication of wild birds in a local enzootic cycle that could allow maintenance and amplification of the virus before and during the outbreak. Findings of past exposure of migratory birds to WNV upon their arrival in Greece during autumn migration, suggest avian species with similar migration traits as candidates for the introduction of WNV into Greece. The possibility that an endemic circulation of WNV could have caused the outbreak, after an amplification cycle due to favorable conditions cannot be excluded.

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3.1 Background

West Nile virus (WNV) is a flavivirus of major public health concern for the last 2 decades, as associated disease outbreaks are increasing worldwide. The main route of infection is through the bite of infected mosquitoes; humans and horses develop viremia levels of low magnitude and short duration, insufficient to re-infect mosquitoes, and thus do not serve as amplifying hosts for WNV in nature [1]. On the contrary, various avian species develop viremia levels sufficient to infect mosquitoes and even bird to bird transmission of the virus by direct contact has been reported [2]. Hence, WNV is maintained in an enzootic cycle with birds being the amplifying hosts and ornithophilic mosquitoes, especially of the Culex species, the main vectors. Moreover, local movements of resident birds and long-range travel of migratory birds may both contribute to the spread of WNV [3,4].

In 2010, a major outbreak of WNV human infections occurred in northern Greece, with 262 laboratory-confirmed cases and 35 deaths [5]. Although WNV neutralizing antibodies had been detected in northern Greece since 2007, the first WNV lineage 2 (L2) strain was obtained from pools of Culex mosquitoes (strain Nea Santa-Greece-2010) in 2010 [6]. At the same time our team detected a similar L2 strain in a Eurasian magpie (strain magpie-Greece/10), as has been reported [7].

For the purposes of our participation in an FP7 EU research project ("WildTech"), wild bird samples that have been collected by the Hunting Federation of Macedonia and Thrace since 2009 were used for serological and molecular surveillance regarding exposure to various pathogens. The objective of this study was to detect possible exposure of wild birds to WNV prior to and during the outbreak. In addition we further investigated the detected WNV magpie strain for important virulence markers. These markers have been recognized to be a prerequisite for the development of viremia levels in wild birds necessary for them to be considered amplifying hosts.

3.2 Findings

Our team conducted a serological and molecular surveillance in serum and tissue samples of wild birds hunter- harvested in 2009–2010 and 2010–2011

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official hunting seasons (from 20 August until 28 February of the following year). Samples were collected at the epicentre of the 2010 outbreak (Figure 1) in central Macedonia by members of the Hunting Federation of Macedonia and Thrace from species considered quarry according to Greek legislation. Samples from all different species were not available for both periods or from every sampling site. All sampling sites are in flying distance of avian species and no safe conclusions can be drawn regarding viral dispersion in the area between the hunting seasons. This area is characterized by mosquito-abundant water-lands and four major rivers which converge into a common delta, a well-known resting and breeding ground for migratory birds. The study was focused on hunter- harvested resident and migratory avian species suspected to play a role in WNV local circulation, maintenance and dispersion. Members of the Corvidae family, like Eurasian magpies (*Pica pica*) and hooded crows (*Corvus cornix*) were prioritized; these corvid species are resident, with a wide daily dispersal range of up to 20 km, social, roosting in large colonies and abundant in both wetlands and urban areas [8]. Turtle doves (Streptopelia turtur) were also targeted; they are suspected to be a principal introductory host of WNV via their migration routes, as the virus has been isolated from actively migrating turtle doves [9]. Migratory waterfowl like the mallard ducks (*Anas platyrhynchos*) have been found to carry WNV antibodies and recent experimental studies have also proven that Anseriformes may be able to function as carriers of WNV [10].

Serum and tissue samples from 295 hunter-harvested birds belonging to the above 4 avian species were collected.

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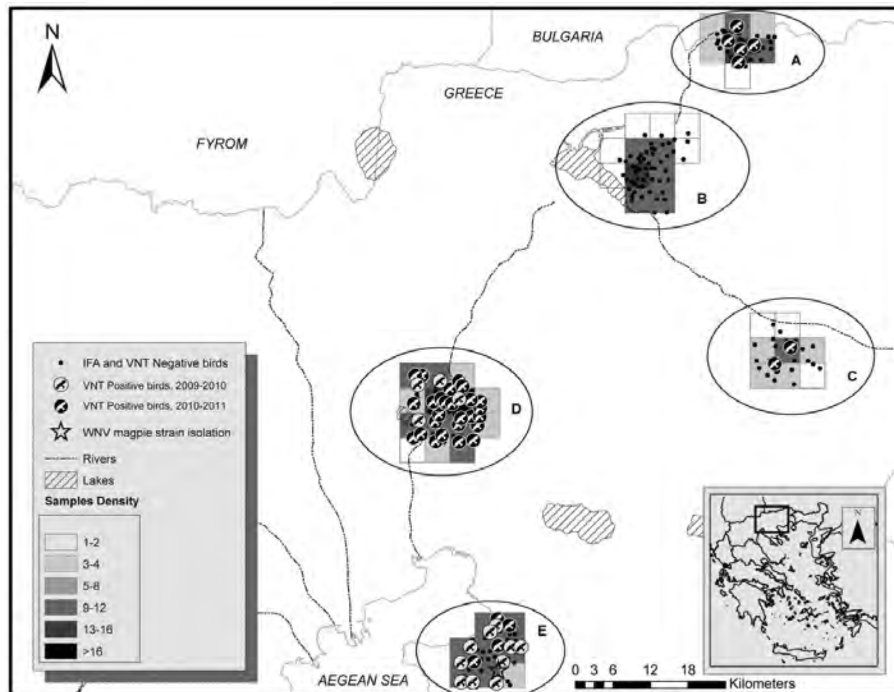

Figure 1 Map of the epicentre of the WNV outbreak, Greece 2010. Map of the epicentre of the outbreak where most of the human West Nile Virus cases occurred during 2010 (Central Macedonia, Northern Greece). The area is characterized by wetlands and is considered a well-known resting and breeding area for migratory birds. Sites A-E refer to bird sampling sites.

Serological screening was performed with an indirect immunofluorescence assay (IFA) test kit (EUROIMMUN) with slight modifications of manufacturers' instructions, to detect avian WNV antibodies, as previously described; the determined cut-off value of 1:30 was used and a (FITC)-labelled goat anti-bird antibody was applied (Bethyl Laboratories Inc.) [11]. Positive results were verified by a micro-virus neutralization test (VNT), as previously described [12].

Serological results are summarized in Table 1 and VNT titres are presented in Table 2. Seventy samples (23.7%) were IFA-positive, 53 of the IFA-positive samples were confirmed by micro-VNT test. WNV- neutralizing antibodies were detected in 14 resident corvid samples hunter-harvested in the 2009/2010 hunting season; six seropositive corvids were collected in October 2009, indicating an avian exposure to WNV at least 8 months prior to the human outbreak. Presence of WNV-neutralizing antibodies in corvids sera collected in both 2009/2010 and 2010/2011 hunting seasons indicates a continuous

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maintained circulation and presence of WNV in the area for the referred period, as hunter- harvested corvids are mainly < 1 year old, born in February-March of the same year.

Seven sera of turtle doves hunter-harvested upon the days of their arrival in resting areas (sites A and C, Figure 1) during autumn migration were found positive for WNV-neutralizing antibodies. Three of these 3 were juvenile, born probably in breeding areas of origin in the same year (central Europe). None of the 55 sera of mallard ducks hunter harvested near the artificial Lake Kerkini, a premier birding site (site B, Figure 1), were found to contain WNV-specific antibodies.

Molecular screening from pools of selected tissue samples (liver, heart and kidney, known to be suitable samples for WNV detection especially in asymptomatic birds) was performed as described previously [7]. A positive WNV L2 PCR product was obtained from a magpie hunted in the area of the human outbreak in September 2010 that was, as reported, similar to the one derived from pools of mosquitoes in the same area [6] and showed the highest sequence similarity to strains derived from birds of prey in Austria in 2008–09 as well as in Hungary in 2004 [13,14]. In this study, further molecular investigation of the magpie WNV strain was performed, regarding important virulence markers. RT-PCR and a subsequent sequencing analysis was employed for the amplification of a 270-nt Envelope (E) protein genomic region, using previously described primers [15]. Molecular investigation of a 401-nt NS3 genomic region of the magpie strain using a previously established PCR protocol, was also performed [16].

The N-linked glycosylation motif (N-Y-T/S) at residues 154–156 of the E protein is present in the magpie strain; E protein glycosylation is considered a prerequisite for the development of the necessary viremic levels in avian blood (> 10^5 PFU/ml) that allow efficient transmission of WNV Lineage 1 from avian hosts to mosquitoes [17]. A nucleotide mismatch was revealed at nt position 624 of E gene (G-C transversion, synonymous SNP) by pair- wise alignment between the present study magpie WNV strain and the mosquitoes WNV strain isolated one month earlier [18]. The E genomic region sequence from the Greek magpie isolate was deposited in GenBank under accession no JN809470. A

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phylogenetic tree was constructed using MEGA 5.0 [19]. Neighbour-joining analysis of genetic distances in the 270-nt E genomic region of WNV strains (Figure 2) displayed close phylogenetic relationship between the Greek WNV strains, a Hungarian WNV strain isolated from a goshawk in 2004 and South African WNV lineage 2 strains.

Molecular investigation of a 401-nt NS3 genomic region of the magpie strain revealed the presence of proline at the 249 aa position of NS3 gene, a mutation

Table 1 Summary of West Nile virus serological results among hunter-harvested wild birds, Greece 2009-2011

Species	Sampling site*	Total no. of bird samples	Hunting season 2009-2010		Hunting season 2010-2011		Total	
			IFA pos./ neg.	VNT pos. (n)†	IFA pos./ neg.	VNT pos. (n)	IFA pos./ neg.	VNT pos. (n)
Pica pica	D	90	5/5	3 (5)	34/46	26 (34)	39/51	29 (39)
	E	58	10/24	8(10)	4/20	3 (4)	14/44	11 (14)
Corvus cornix	D	11	1/3	1 (1)	3/4	2 (3)	4/7	3 (4)
	E	12	3/5	2(3)	1/3	1 (1)	4/8	3 (4)
Anas platyrhynchos	B	55	0/25	-	0/30	-	0/55	-
Streptopelia turtur	A	45	-	-	6/39	5 (6)	6/39	5 (6)
	C	24	-	-	3/21	2 (3)	3/21	2 (3)
Total		295	19/62	14 (19)	51/163	39 (51)	70/225	53 (70)

Prevalence of West Nile virus antibodies among wild birds hunter-harvested at the epicenter of a major human outbreak of the disease (central Macedonia, northern Greece). All IFA positive samples were further verified by VNT.
*Sampling Sites A-E correspond to the indicated areas of Figure 1.
†n number of samples tested positive by IFA.

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Table 2 Virus neutralization titers of avian sera samples considered positive

Sampling site	Species	Number tested	1/10	1/20	1/40	1/80	1/160
A	*Streptopelia turtur*	5	1	1	1	2	-
C	*Streptopelia turtur*	2	-	1	-	-	1
D	*Pica pica*	29	4	14	5	3	3
	Corvus cornix	3	-	-	2	1	-
E	*Pica pica*	11	3	6	-	1	1
	Corvus cornix	3	-	1	1	-	1

West Nile virus neutralizing antibody titers measured in VNT positive samples (threshold 1/10 for a sample to be considered positive) of each sampling site as indicated in Figure 1.

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related to increased viremia potential and virus transmission rates in corvids for L1 strains [20]. This mutation was also present in the other WNV L2 strains isolated in Greece [18,21]. All other WNV L2 strains isolated worldwide have histidine at this position and have not been related with major human outbreaks [18]. The NS3 genomic region sequence from the magpie isolate was deposited in GenBank under accession no. JN809471.

3.3 Conclusions

This is the first report regarding extensive exposure of wild birds to WNV in Greece prior to the 2010 human outbreak. Findings of high virus neutralization titres in many samples suggests a possible recent exposure to WNV rather than cross reaction to other flaviviruses such as USUV. In addition, our employed RT-PCR designed to amplify a wide range of mosquito-borne flaviviruses did not give any positive result other than WNV. Resident corvids hunter-harvested in the epicentre of the outbreak have been exposed to WNV at least eight months before the first human cases were reported. Thus an active wildlife surveillance system for emerging infectious diseases would predict the mosquito-wild birds WNV circulation and the possible emergence under appropriate conditions that caused the major outbreak in humans. Genetic determinants of increased virulence were present in the WNV strain isolated from the magpie that further support this finding. However, different findings have also been reported regarding the NS3249 mutation suggesting that this mutation may not be sufficient to enhance virulence for any given WNV strain [22]. Thus, experimental infection studies and pathogenicity assessment will provide more solid conclusions. Furthermore, genetic variation was observed in the related Greek strains isolated from different hosts; this supports the hypothesis of the quasispecies structure and possible process of adaptation to local transmission of the virus [23]. Even though our study confirmed the presence of important genetic virulence markers in the magpie strain, clinical signs suggestive of encephalitis or dead birds were not reported from any of the hunters. The hunters had been briefed and were instructed to report any such observations. Wild birds in Greece do not seem to be susceptible to WNV even though the virus was able to cause a major human outbreak; this further supports the hypothesis that birds in Europe may have an innate immunity due

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to the ancestral co evolution and long association between WNV and avian hosts in the Old World [8]. Findings of WNV neutralizing antibodies in migratory hunter-harvested turtle doves (some juvenile and with high VNT titres) upon their arrival in resting areas of Greece during their autumn migration from breeding areas of central Europe to wintering areas of Africa, indicates exposure probably in the areas of their origin. This finding in addition to the molecular similarity of WNV strains isolated in Greece with strains isolated in previous years in Austria and Hungary [13,14] suggests avian species with similar migration traits as candidates for possible introduction of WNV L2 strain into Greece in previous years from central Europe. The detection of a WNV L2 infection in Italy in 2011, with the isolated strain being closer phylogenetically to the Hungarian and Austrian L2 strains rather than African strains supports this hypothesis [24], especially as Italy and Greece are stopover areas during autumn migration of various avian species from central Europe to Africa [25]. Of course, we cannot exclude the possibility of infection of migratory birds at the stopover areas or that an endemic circulation of WNV could have caused the outbreak after an amplification cycle due to favourable conditions present in the epicentre of the outbreak. Results of this study strengthen the need for a continuous active serological and molecular surveillance system regarding WNV and other flaviviruses, which may provide timely information regarding virus introduction and circulation, further dispersion or introduction of new strains.

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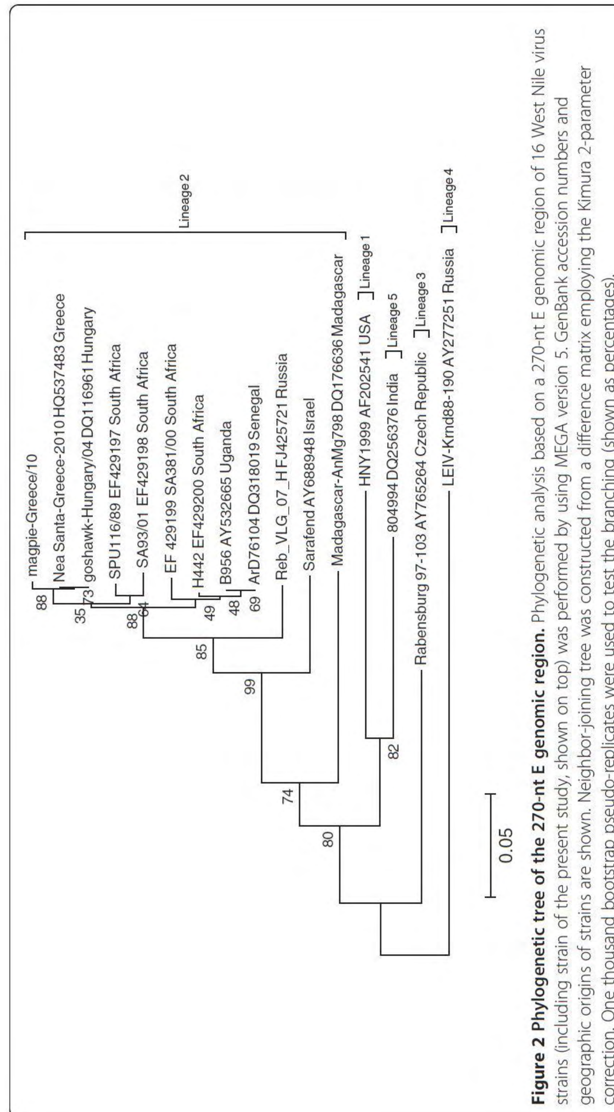

Figure 2 Phylogenetic tree of the 270-nt E genomic region. Phylogenetic analysis based on a 270-nt E genomic region of 16 West Nile virus strains (including strain of the present study, shown on top) was performed by using MEGA version 5. GenBank accession numbers and geographic origins of strains are shown. Neighbor-joining tree was constructed from a difference matrix employing the Kimura 2-parameter correction. One thousand bootstrap pseudo-replicates were used to test the branching (shown as percentages).

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CHAPTER 4

EXPOSURE OF EURASIAN MAGPIES AND TURTLE DOVES TO WEST NILE VIRUS DURING A MAJOR HUMAN OUTBREAK, GREECE, 2011

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Summary

A major number of West Nile virus (WNV) infections in humans occurred in 2010 in northern Greece, with 262 laboratory confirmed cases. In 2011, fewer cases were reported, but the pattern was more dispersed throughout the Greek mainland. Isolated strains were similar to lineage 2 strains detected in previous years in Austria and Hungary from birds of prey. We conducted a serological surveillance study on hunter-harvested wild birds, to determine possible exposure of avian species during the current outbreak. Serum samples from a total of 113 Eurasian magpies and 85 turtle doves (abundant resident and migratory avian species, respectively, with potential roles in WNV epidemiology) were tested. These birds were hunter-harvested during 2011 from various prefectures both affected and not affected by the WNV outbreak in Greece. Sera were tested for the presence of WNV IgG antibodies by indirect immunofluorescence assay (IFA). Verification of positive results by a micro-virus neutralization test (VNT) was also performed. A total of 23 out of 113 (20.4%) Eurasian magpies and 6/85 (7.1%) turtle doves were found positive. Results showed association of human cases with wild birds' exposure to the virus; no avian sera were found positive in prefectures not affected by the WNV outbreak. In contrast, positive avian sera were found in every prefecture that human WNV cases occurred in 2011. High seroprevalence in Eurasian magpies suggests high activity of WNV in the areas. Findings of past exposure of migratory birds like turtle doves to WNV upon their arrival in resting areas in Greece suggest various avian species with similar migration traits as target species for viral isolation studies, as they can be considered candidates for the introduction of WNV lineage 2 in Greece from Central Europe.

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4.1 Introduction

In 2010, a major number of West Nile virus (WNV) human infections occurred mostly in northern Greece, with 262 laboratory-confirmed cases, of which 191 presented with neuroinvasive symptoms and 35 led to death (HCDCP 2010). WNV lineage 2 sequences were obtained from pools of Culex mosquitoes (strain Nea Santa-Greece-2010) (Papa et al. 2011) and a Eurasian magpie (strain magpie-Greece/10) (Valiakos et al. 2011). Significantly fewer cases were reported in 2011; from the beginning of 2011 until mid-September, only 74 laboratory-confirmed cases of WNV infection were reported, of which 57 presented with neuroinvasive disease and five led to death (HCDCP 2011). However, many of the 2011 cases were observed in areas not previously affected; a southwards dispersion of WNV was observed to new areas which are characterized by warmer climate, milder winters and still major mosquito-abundant wetlands. Some were adjacent to areas affected in 2010 whilst others were in new distant areas. A WNV lineage 2 strain was reported in seroconverted sentinel chickens in July 2011 and in one pool of Culex pipiens mosquitoes this year in Central Macedonia (Chaskopoulou et al. 2011; Danis et al. 2011).

An ongoing serological surveillance study regarding exposure of wild birds to a number of different pathogens, including WNV, is being conducted that tests avian samples for the purposes of an EU research project (WildTech 2010). Regarding WNV, the objective of our study is to detect possible exposure of wild birds to the virus in prefectures affected by the outbreak and in prefectures that were not affected.

4.2 Methods and results

Our team has conducted a serological surveillance study in hunter-harvested migratory and resident wild birds in 2011. Eurasian magpies (Pica pica) were targeted, which are resident corvids, with a wide daily dispersal range of up to 20 km and roost in large colonies in both wetlands and urban areas (Jourdain et al. 2008; Reiter 2010). Turtle doves (Streptopelia turtur) were also targeted by our study as they constitute a major representative of migratory birds in Greece and are suspected to be one of the principal introductory hosts of WNV

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via their migration routes worldwide, as the virus has been isolated from actively migrating turtle doves (Ernek et al. 1977).

Serum samples from a total of 113 Eurasian magpies and 85 turtle doves were collected during 2011 from various prefectures, some of which were affected by the WNV outbreak (n=6) while others were unaffected (n=3). Most birds were hunter-harvested in late August to early September 2011 (start of the 2011–2012 hunting season according to the Greek law) (YPEKA 2011). Some sera collected in February 2011 (end of 2010–2011 hunting season) in prefectures of Larissa and Attica were also tested. Sera were tested for the presence of WNV specific antibodies by indirect immunofluorescence assay (IFA); commercially available slides (EUROIMMUN®; Luebeck, Germany) were employed, as described previously, and the determined cut-off value of 1:30 was used (Ziegler et al.2010). Each sample was placed in a single substrate containing both WNV infected and non-infected cells in order to avoid false positive results. Verification of positive results by a micro- virus neutralization test (VNT) was performed, as previously described, in dilutions of 1/10 and 1/20; titres of 10 or higher were determined to be only detected in sera from WNV-infected individuals and not to different flaviviruses (Figuerola et al. 2007).

Results are shown in Fig. 1. A total of 23/113 (20.4%) Eurasian magpies and 6/85 (7.1%) turtle doves were found IFA/VNT positive. To gain further insight into the potential cross-reactivity of other circulating flaviviruses, namely, TBEV in Greece (Pavlidou et al. 2007), IFA/VNT positive samples were further tested using commercial TBEV IFA slides (EUROIMMUN®). No positive results were observed. Finally, RT-PCR was employed in pools of selected tissues (heart, kidney, liver) from all seropositive and 18 seronegative birds, as previously described (Valiakos et al. 2011; Weissenbock et al. 2002). No WNV RNA was detected in these samples.

No avian sera were found positive in prefectures not affected by the human WNV outbreak from which samples were available in 2011 (prefectures of Florina, Evros and Arcadia with a total of 0/52 positive avian sera). In contrast, positive avian sera were found in every prefecture in which human WNV cases occurred in 2011 (prefectures of Serres, Thessaloniki, Trikala, Larissa and

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Karditsa with a total of 29/127 positive avian sera) except for Attica; however this prefecture was not affected by the outbreak of 2010 and available avian sera were collected in February 2011 (first human cases in the area were reported in July 2011). On the other hand, in prefecture of Larissa, sera collected in February 2011 were found positive; human cases have been also reported here in August 2010 (HCDCP 2010).

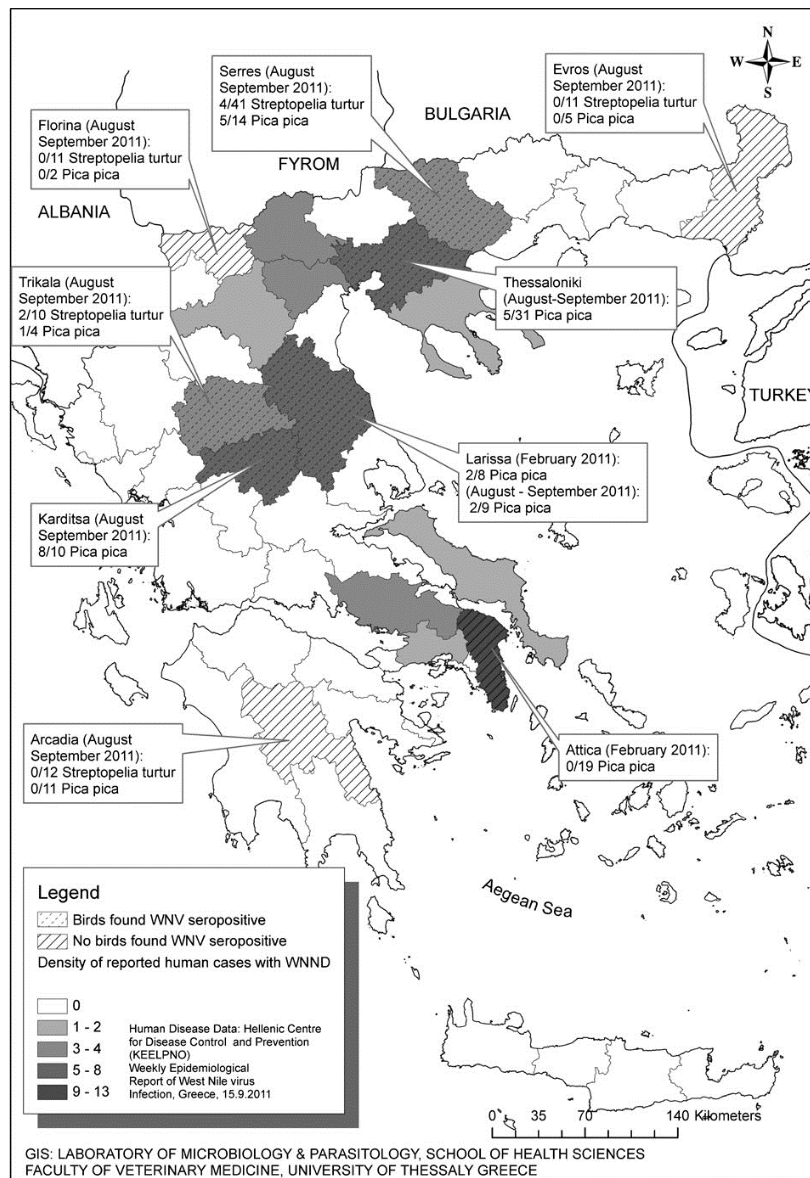

Figure 1. Occurrence of WNV human cases and results of serologic surveillance in wild magpies and turtle doves (Greece, February–September 2011). Map of Greece showing prefectures of Greece where occurrence of human cases of WNV was observed until 15 September 2011, in relevance to

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tested avian samples. Number of tested positive/total number of tested avian samples per species is shown for each prefecture for which avian sera were available for our study.

4.3 Discussion

First serological results showed association of human cases with wild birds' exposure to the virus; none of the sera derived from birds hunter-harvested in prefectures unaffected by the WNV human outbreak was found positive. On the contrary, birds were found to be exposed to WNV in every prefecture that reported human cases and from which avian sera were available ($\chi^2 = 12.5$, df = 1, $P < 0.001$). High seroprevalence of resident wild birds (Eurasian magpies) suggests a high activity of WNV in the affected areas. Detection of WNV RNA in tissues of a Eurasian magpie hunter-harvested in September 2010 (Valiakos et al. 2011), in pools of mosquitoes 1 month earlier (Papa et al. 2011) as well as in sentinel chickens in July 2011 (Chaskopoulou et al. 2011), further supports this finding, indicating a recent bird–mosquito circulation rather than a past exposure to WNV at least in the area of northern Greece. Hence, our results further strengthen previous studies that suggested magpies as sensitive indicators of WNV enzootic activity in an area (Jourdain et al. 2008). Exposure of migratory wild birds like turtle doves creates concerns about the origin and/or further dispersion of the virus. Turtle doves are one of the species known for their ability to travel long distances (over 1,000 km) during their migrations with an average speed of 90 km/h. During their autumn migration, turtle doves from the Balkan countries can fly over the Mediterranean basin in just one night to reach their wintering sites of sub-Saharan Africa. However, turtle doves from Central European countries (Hungary, Austria, Poland, etc.) are not able to pass over the Mediterranean basin at once, but stop at several resting areas in Greece to replenish which has been proven by recovery of ringed birds projects (Akriotis and Handrinos 2004; Bankovics 2001). Even though WN viremia levels capable of mosquito infection last for only a few days in birds, the migration speed of turtle doves as well as the previous examples of isolation of WNV from actively migrating turtle doves (Ernek et al. 1977) allow their implication in WNV epidemiology. In our study, WNV-reactive antibodies were detected in sera of migratory turtle doves which were hunter-harvested on the

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day of their arrival at resting areas of Greece during their autumn migration from Central Europe to wintering areas of Africa. This demonstrates past exposure to WNV probably to the areas of their origin. The high molecular similarity of WNV strains isolated from various species in Greece (Papa et al. 2011; Valiakos et al. 2011; Chaskopoulou et al. 2011) Austria and Hungary (Wodak et al. 2011; Bakonyi et al. 2006) suggests a south- wards dispersion of WNV from Central Europe which may have been caused through the migration routes of migratory avian species with similar migration traits (turtle doves, quails, orioles, etc.) (Jourdain et al. 2007). Isolation of WNV strains from birds of these species will provide even more evidence to support this hypothesis. Furthermore, exposure of birds of these species to the virus while resting in Greece would create further concerns about virus dispersion.

The hunters were briefed on signs of encephalitis in birds, and were instructed to report any such observations. However, no nervous signs in wild birds or findings of dead birds were reported by any of the hunters. Wild birds in Greece do not seem to be susceptible to the circulating WNV lineage 2 strain that causes major human outbreaks; this further supports the hypothesis that birds in Europe may have an innate immunity due to the ancestral co-evolution and thus long association between the virus and its avian hosts in the Old World (Reiter 2010).

No sera of birds hunter-harvested in Attica on February 2011 were found positive. Even though the sample size is small, we may hypothesize that the introduction of the virus occurred later in the area near the capital of Athens, probably justifying the relatively small outbreak in a high population area. Attica is characterized by mosquito abundant wetlands known to serve as resting areas for migratory birds. Hence, concerns arise regarding possible overwintering and following extended amplification of WNV leading to a potential larger outbreak in the next year.

Further serological and molecular surveillance of resident and migratory birds will provide further information regarding possible virus origin and further dispersion of the isolated WNV strains or even introduction of new strains.

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CHAPTER 5

USE OF WILD BIRD SURVEILLANCE, HUMAN CASE DATA AND GIS SPATIAL ANALYSIS FOR PREDICTING SPATIAL DISTRIBUTIONS OF WEST NILE VIRUS IN GREECE

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Summary

West Nile Virus (WNV) is the causative agent of a vector-borne, zoonotic disease with a worldwide distribution. Recent expansion and introduction of WNV into new areas, including southern Europe, has been associated with severe disease in humans and equids, and has increased concerns regarding the need to prevent and control future WNV outbreaks. Since 2010, 524 confirmed human cases of the disease have been reported in Greece with greater than 10% mortality. Infected mosquitoes, wild birds, equids, and chickens have been detected and associated with human disease. The aim of our study was to establish a monitoring system with wild birds and reported human cases data using Geographical Information System (GIS). Potential distribution of WNV was modelled by combining wild bird serological surveillance data with environmental factors (e.g. elevation, slope, land use, vegetation density, temperature, precipitation indices, and population density). Local factors including areas of low altitude and proximity to water were important predictors of appearance of both human and wild bird cases (Odds Ratio = 1,001 95%CI = 0,723–1,386). Using GIS analysis, the identified risk factors were applied across Greece identifying the northern part of Greece (Macedonia, Thrace) western Greece and a number of Greek islands as being at highest risk of future outbreaks. The results of the analysis were evaluated and confirmed using the 161 reported human cases of the 2012 outbreak predicting correctly (Odds = 130/31 = 4,194 95%CI = 2,841–6,189) and more areas were identified for potential dispersion in the following years. Our approach verified that WNV risk can be modelled in a fast cost-effective way indicating high risk areas where prevention measures should be implemented in order to reduce the disease incidence.

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5.1 Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus with increasing numbers of reported human disease cases worldwide. In Europe, cases of WNV associated diseases have been reported in several countries in the European Union and in bordering Non- E.U. countries. The largest ongoing European outbreak has been observed in Greece, with more than 524 confirmed cases of human infection and 60 deaths reported since 2010 [1] (Figure 1). Many studies have associated the presence of specific environmental factors with areas at high-risk for WNV transmission in the USA [2–5] and Europe [6,7]. Tachiiri et al. (2006) developed a model using basic geographic and temperature data to assess WNV risk in British Columbia [8]. Ruiz et al. (2004) used several factors related to the physical environment such as elevation range, physiographic region, and percentage of vegetation cover to determine WNV risk during an outbreak in the Chicago area in 2002 [9]. Methods that have been used in WNV risk modelling include non-linear discriminant analysis [10], logistic [11] or multiple regression models [12] (differential and difference

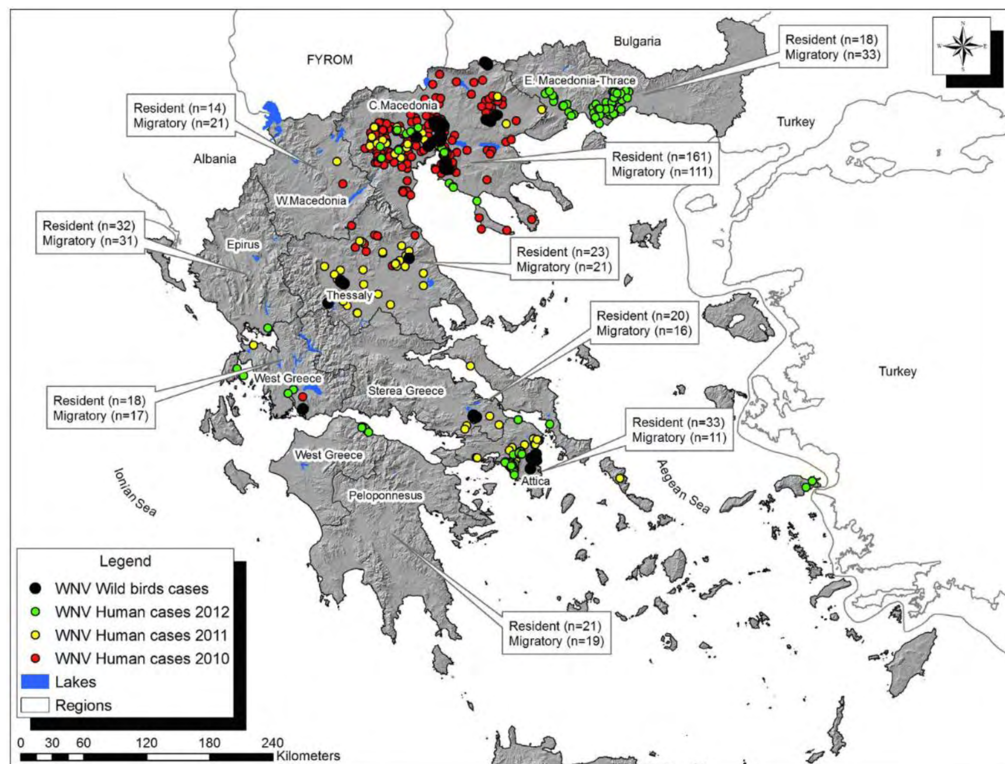

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Figure 1. Map of Greece showing WNV laboratory-confirmed human cases and seropositive resident wild birds, 2010–2012. Map of Greece showing the distribution of WNV laboratory-confirmed human cases and seropositive resident wild bird samples for the 2010–2012 period. Red, yellow and green dots indicate human cases reported in 2010, 2011 and 2012 respectively. Black dots indicate seropositive resident wild birds detected during the same period. Text boxes refer to available avian samples (resident and migratory) per each region.

equation modelling [13,14] and cluster analysis [15]. Predictive modelling with Geographic Information System (GIS) can be used to analyse environmental determinants of WNV transmission and determine high risk areas. Most previous WNV risk analyses utilized spatial statistical techniques (mapping clusters, geographic distribution, spatial relationships-regression models) to correlate environmental, climatic and socioeconomic factors with WNV prevalence [2,4,5,9,11,16]. The geographical position of Greece in the Mediterranean peninsula makes it an important transit zone for migratory birds [17]. Greece hosts a wealth of biological diversity, one of the richest in Europe and the Mediterranean.

The main objective of this study was to correlate serological data of exposure of wild birds to WNV and reported human cases data during the Greek outbreak with potential environmental risk factors within a GIS, in order to construct predictive maps identifying areas at risk from further spread. We test the predictive power of the models against recent outbreak data and identify high risk areas for the application of targeted, timely and cost-effective prevention measures such as surveillance, mosquitoes control and campaigns to increase public awareness of the disease.

5.2 Materials and Methods

Study Area

The study area comprised the entire country of Greece. Greece occupies the south-eastern part of Europe with a total area of 131,990 km2. Eighty percent of Greece consists of mountains; the country is characterized by a large climatic diversity (29 climatic zones according to the Thorn Waite classification), by its

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extensive coastline of about 15,000 km and many island complexes in the Archipelagos of Aegean Sea and the Ionian Sea. Climatic conditions of the country are typical Mediterranean: Summer is hot and dry while winter is usually mild. Rain mostly falls in autumn and winter.

WNV Human Cases Data

Reported human WNV cases in Greece (2010–2012) were provided by HCDCP. Most cases were serologically confirmed by the presence of IgM antibodies in the serum and/or the cerebrospinal fluid. Residential address of each human case was used for geocoding and mapping the cases.

Wild Birds Surveillance

A total of 620 avian serum samples were obtained from wild birds hunter-harvested by members of the Greek Hunting Federation of Macedonia and Thrace, from species considered quarry during the 2009/2010, 2010/2011 and 2011/2012 hunting seasons (from 20 August until 28 February the following year), according to the prerequisites of the Greek Legislation. All available samples were obtained from mainland Greece, opportunistically collected during regular hunting activities; samples were available from all 9 mainland regions of Greece (Table 1). Sampling effort was distributed in mainland Greece, avoiding cluster sampling biases, with the exception of the Central Macedonia region, the epicentre of the outbreak, during which a large number of samples were provided. Data on bird specimens that tested positive for WNV during the study were located in the field using handheld Global Positioning System (GPS) units or located by means of longitude and latitude information provided by samplers. Serological screening was performed as already reported [18–20]; a total of 64 resident wild birds were found positive for WNV antibodies, and were used in the current study (migratory wild birds were also found seropositive, but relevant data was excluded from the analysis, see Discussion).

Environmental Variables

Environmental variables for this study were derived from three main database categories: climate, elevation and land cover data. WorldClim version 1.4 climate data [21] was obtained from the WorldClim website

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(http://www.worldclim.org). WorldClim is a set of global climate layers (climate grids) with a spatial resolution of 1 square kilometre. Topographic variables including altitude, aspect and slope were extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometre (http://srtm.csi.cgiar.org/Index.asp). Land uses were derived from the Corine Land Cover 2000 database (European Environment Agency – EEA, http://www.eea.europa.eu/data-and-maps).

Village and vegetation corrections were digitized from 2007 and 2009 colour orthophotos that were available through Web Mapping Service (WMS) (http://gis.ktimanet.gr). To create environmental layers (n = 37) for the analysis (Table 2), ArcGIS 10.1 GIS software (ESRI, Redlands, CA, USA) was used. GIS layers were created to represent factors like the locations of towns and villages, distance to the nearest village, distance from water presence etc. For many of the above parameters, we calculated neighbourhood statistics for radii of 100, 200, 500 and 1000 m to determine which spatial scale affects the presence of cases most strongly. These data sets were converted to a common projection, map extent and resolution prior to use in the modelling program.

Statistical Analysis

We used data on 2010 and 2011 human cases for the statistical analysis and model building and kept the 2012 cases for verification. A total of 363 human WNV cases have been reported in Greece for the years 2010 and 2011 (262 cases in 2010 and 101 cases in 2011). The available dataset consisted of presence only data (presence: people infected by the virus). For this dataset, as well as the wild birds seroprevalence dataset a number of explanatory variables (n = 37) were collected and constructed, as mentioned previously (Table 2).

Instead of constructing a number of pseudo-absence controls, a methodology which according to the literature has some significant disadvantages for the prediction modelling [22,23], we decided to search for within the presence data variation of the explanatory variables. We clustered the cases using the agglomerative method of Two Step Cluster Analysis, a method which allows for the utilization of both continuous and categorical variables and clusters the

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cases by measuring the log-likelihood distance among them [24]. The Two Step Cluster Analysis allowed us to check for a pattern of the virus among the infected people in 2010, in 2011, and in total. The optimal number of clusters was chosen using the Silhouette coefficient, a measure proposed by Kaufman and Rousseeuw (1990) [25]. The coefficient ranges from 21 to 1 and when its value is closer to 1, the clustering is considered efficient.

Before applying the above cluster method, we checked a number of descriptive statistical measures which describe our data. Although Two Step Cluster Analysis is robust to non-normality [24] we used Factor Analysis in order to reduce the number of available variables and to achieve normality and zero-correlation among explanatory continuous variables. We used the Principal Component Analysis (PCA) as a method of components extraction with rotation method the Varimax method with Kaiser Normalization [26]. Two Step Cluster Analysis was iterated several times using as clustering variables either the components which were extracted by the Principal Component Analysis, or the original variables which were highly correlated with the components. The extracted clusters for humans and the extracted clusters for birds were compared in terms of the variables that are important for clustering.

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Table 1. Available avian samples: species, migratory status and number of samples per region.

Species	Migratory Status	E. Macedonia & Thrace	Central Macedonia	Thessaly	Epirus	West Greece	Peloponnesus	Sterea Greece	Attica	West Macedonia
Pica pica	Resident	11	125	17	20	12	14	13	29	9
Streptopelia turtur	Migratory	8	35	4	11	7	8	7	5	8
Corvus cornix	Resident	4	18	5	8	4	4	6	4	3
Anas platyrhynchos	Migratory	5	26	3	0	0	0	2	0	0
Scolopax rusticola	Migratory	4	12	5	9	3	6	2	0	5
Turdus philomelos	Migratory	7	27	6	8	7	5	4	6	8
Corvus monedula	Resident	3	18	1	4	2	3	1	0	2
Anas crecca	Migratory	9	11	3	3	0	0	1	0	0
Total		51	272	44	63	35	40	36	44	35

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GIS Analysis

Two significant environmental variables were recognized from the statistical analysis (see Results) and were used to measure environmental conditions for the WNV locations of the seropositive wild birds and the human cases dataset. Mahalanobis distance (MD) [27] was used to develop a distance measure

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model for wild birds and predict WNV potential distribution prior to the expansion/outbreak of the 2012 period. We calculated MD with ArcGIS software, based on the values of the two significant variables, allowing us to identify suitable areas for WNV potential distribution and occurrence. Model performance evaluation was conducted with the 2012 reported WNV human cases, as provided by HCDCP.

5.3 Results

Data analysis demonstrates differences between 2010 and 2011 in terms of positive cases in Greece (Table 3). Fewer cases (n = 101) occurred in 2011 and the average case age was 5 years younger compared to 2010 (p-value = 0.024). There was a statistically different distribution in terms of the prefecture of residency of the positive cases, which were found in more southern areas compared to 2010, indicating the pathogen's continued spread in mainland Greece.

Finally, the distribution of the infected individuals in terms of date of infection was different in 2011, where more positive cases were found in July and September, compared to 2010 where the majority of the cases were found in August (64%).

Factor Analysis and Two Step Cluster Analysis revealed that altitude and distance from water were the two variables, among the 37 under study, which clustered significantly the cases. Both variables played a significant role in the clustering procedure. The two variables clustered in a similar way for both humans and birds. For the clustering of human cases, the average Silhouette coefficient was 0.5 which is considered a good clustering value [25]. The same value was achieved for the clustering of seropositive wild birds (Odds Ratio = 1,001 95%CI = 0,723– 1,386). Three clusters were created for humans and birds (Figure 2), sharing the same attributes. In particular, humans' Cluster A and birds' Cluster B share the majority of the positive cases in humans and birds respectively (60%). There seems to be a pattern of WNV in Greece in places with low altitude and small distance from water. There are also two other clusters with lower percentages of cases which show that positive cases are

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also found in places with low altitude and big distance from water (23–24%) and in places with high altitude and small distance from water (almost 17%).

Relevant box-plots (Figure 3) show how well the two variables discriminate in each cluster. A clear separation of the three clusters is seen in both groups of cases.

Regarding the 2010 human cases, clustering showed that low altitude and small distance from water were associated with the majority of the positive human cases as well. A total of 86.6% of the human cases were grouped in cluster A (Figure 2), which shares similar attributes with cluster A of human 2011 cases and cluster B of birds.

The potential geographic distribution of WNV, predicted by GIS and MD based on the attributes of the major clusters of reported human cases of 2011 and seropositive wild birds is displayed in Figure 4. Fragmented high-risk areas were recognized: Most were concentrated in the Macedonian prefecture, in western Greece as well as in Thessaly. Other suitable high risk areas were located along the coast line of the Peloponnese peninsula and Crete. Moreover, many Greek islands have suitable environmental characteristics such as Rhodes, Mytilene, Chios, Samos etc.

In the early transmission period (June 2012) we reported the high–risk areas recognised throughout this study to the Ministry of Public Health and to HCDCP. As already reported, in 2012, a total of 161 laboratory-confirmed human cases were reported. Out of these 161 cases, only 31 occurred far from WNV high-risk areas recognised by our model (Odds = 130/31 = 4,194 95%CI = 2,841–6,189); four (4) human cases out of 5 were reported in recognised high-risk areas while only 1 out of 5 was not. New areas of potential dispersion of the virus are also suggested for the following years in the areas of Thrace, the Peloponnese peninsula and several Greek Islands (Figure 4).

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Table 2. Environmental variables used in the analysis.

Variable	Value	Source
Slope (degrees) at 100-, 200-, 500- and 1000-m radii	X, SD, min, max	DEM
Topographic position index (4 classes)	binary	DEM
Altitude	continuous	DEM
Aspect	N,W,S,E	DEM
Distance from nearest village (m)	continuous	ArcGIS-WMS
Distance from water (m)	continuous	ArcGIS-DEM
Habitat types (4 classes: Forests, cultivations, etc.)	Categorical transform to continuous	ArcGIS-Corine LC (EEA)
19 Climatic variables (Temperature 11 indices, Precipitation 8 indices)	continuous	World Clim Database
NDVI (Normalized Difference Vegetation Index) 12 indices	continuous	World Clim Database
Population density	continuous	GEoDatabase

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5.4 Discussion

Humans and other mammals, particularly horses, are alternative hosts for WNV; the main route of infection is through the bite of an infected mosquito. Most human infections remain asymptomatic with WNV fever developing in approximately 20% of infected people and West Nile neuroinvasive disease in 1% [28]. Horses and humans develop low viremic loads (<10^5 PFU/ml) of short duration and thus are considered dead-end hosts for WNV [29]. In contrast, various migratory and resident avian species develop high viremic loads, sufficient to infect feeding ornithophilic mosquitoes [30]. Hence, the WNV life cycle is maintained with birds being the main amplifying hosts and mosquitoes the main vectors. Moreover, local movements of resident birds and long-range travel of migratory birds may contribute to pathogen dispersion [31,32]. In southern France, WNV was detected in late summer of 2000 and 2004. Migratory passerines were found with higher prevalence of WNV neutralizing antibodies (7.0%) than resident and short-distance migratory passerines (0.8%), suggesting exposure to WNV or a related flavivirus during overwintering in Africa [33]. Additionally in Spain it was found that Trans-Saharan migrant species had both higher prevalence and antibody titres than resident and short-distance migrants [34].

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Table 3. Descriptive statistics of positive human cases 2010–2011.

Variable	2010	2011	Total
Positive cases	262	101	363
Age*	67.5 (sd = 16.9)	62.4 (sd = 19.9)	66 (sd = 17.9)
Sex	47.7% women	36.6% women	44.6% women
Prefecture of residency+	31% Thessaloniki	30% Attiki	25% Thessaloniki
	22% Pella	15% Larissa	18% Pella
	19% Imathia	10% Thessaloniki	15% Imathia
Date of infection	11.8% July	20.8% July	
	63.8% August	45.5% August	
	24.0% September	30.7% September	
	0.4% October	3% October	

*Statistically significant reduction in 2011 (5 years) (p-value = 0.024 < 0.05).
+Independence between 2010 and 2011 (p-value = 0.000 < 0.001).
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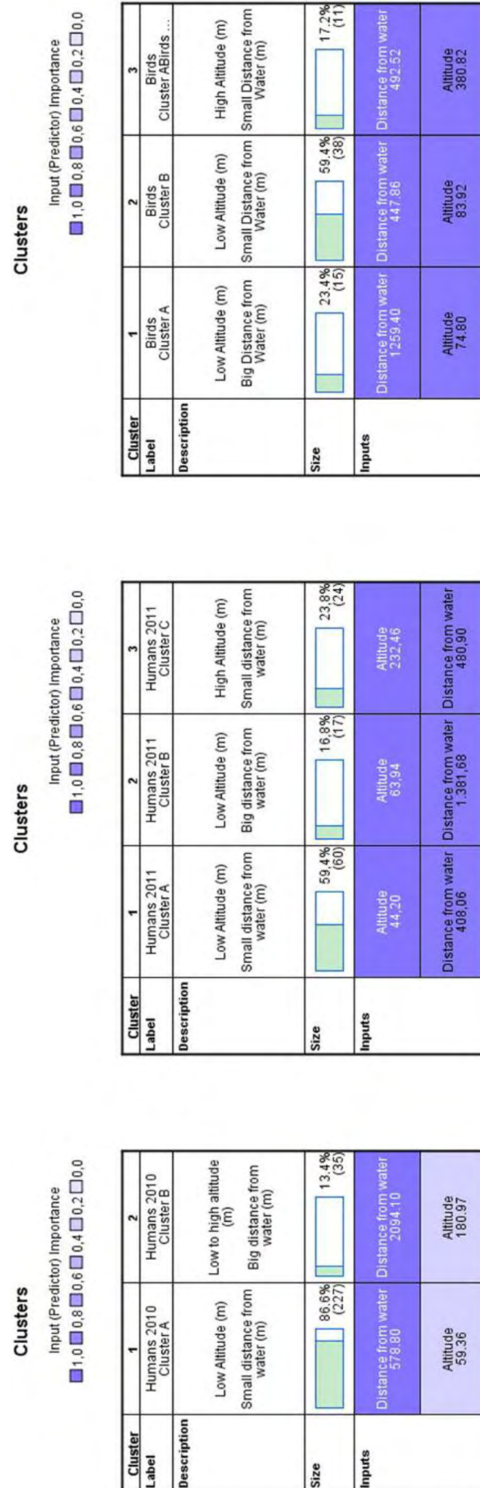

Figure 2. Clusters of human cases of seropositive wild birds and reported human cases of 2010–2011. Clusters of WNV reported human cases of 2010–2011 and seropositive wild birds, according to attributes of altitude and distance from water. Mean values of the two variables are presented under each cluster.

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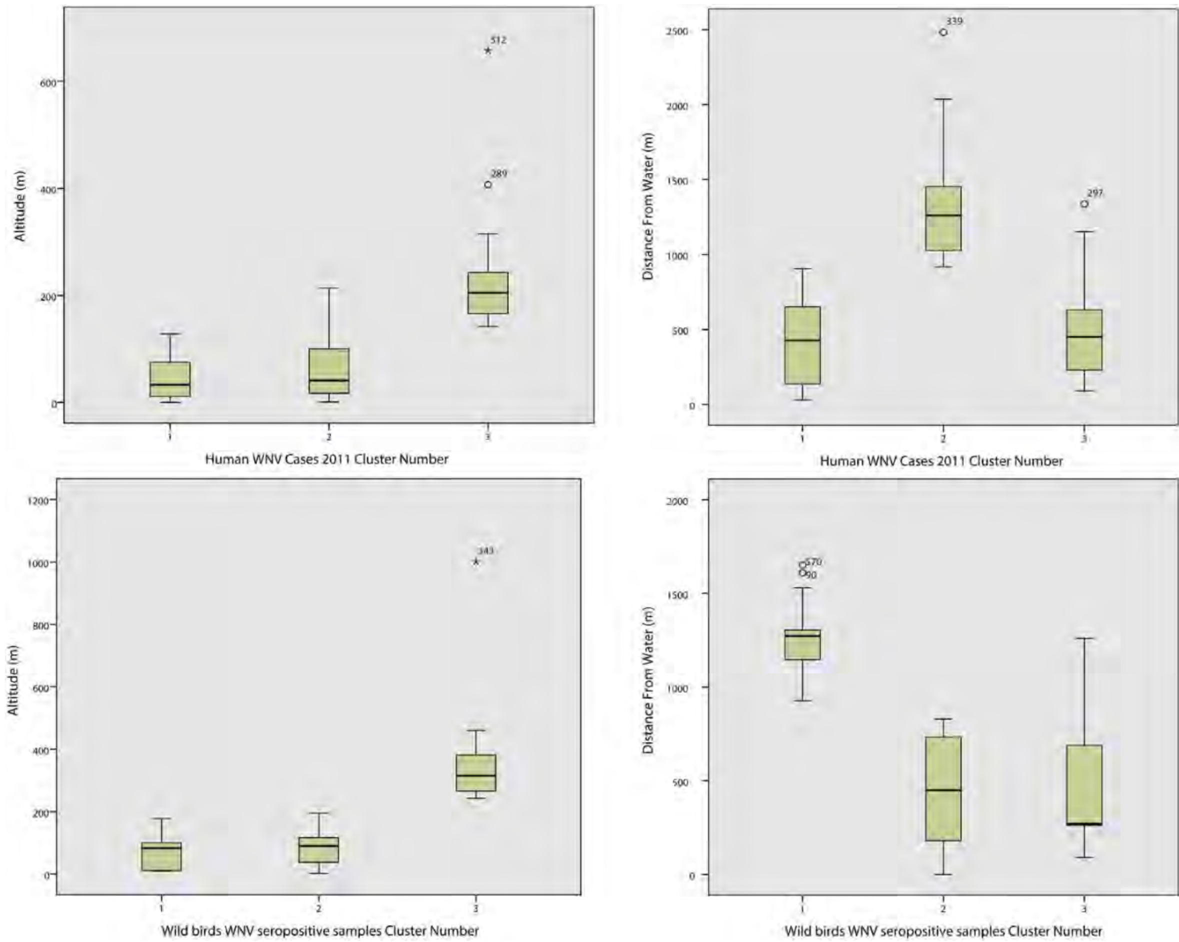

Figure 3. Box-plots of range and altitude. Box-plots displaying range of altitude (left) and distance from water (right) in the three clusters of humans 2011 WNV positive cases and seropositive wild birds.

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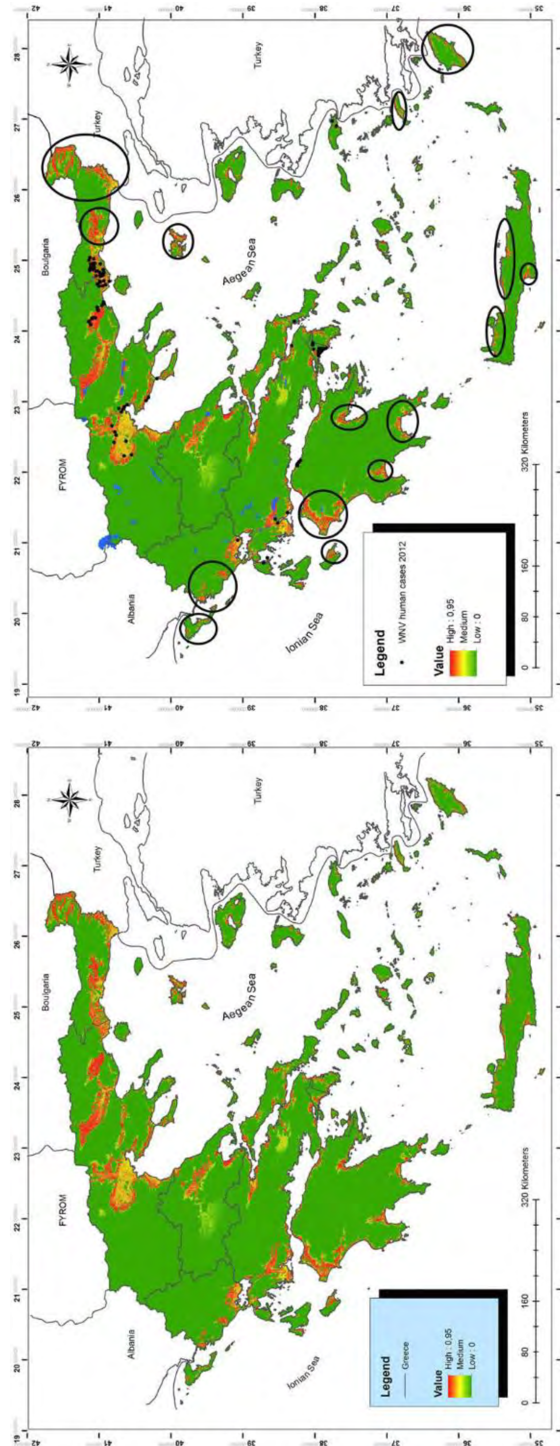

Figure 4. Map of Greece showing potential geographic distribution of WNV. Map of Greece showing potential geographic distribution of WNV, predicted by GIS and MD based on the attributes of the major clusters of reported human cases of 2011 and seropositive wild birds (low altitude, small distance from

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water). Black dots indicate reported human cases in 2012. Black circles indicate suggested high-risk areas for WNV further dispersion in following years.

In Greece, the disease first appeared in Macedonia prefecture in 2010, with 262 confirmed cases and 35 deaths, and it subsequently spread through mainland Greece in the following years. More specifically, in 2011, the outbreak expanded southwards to central Greece with 101 confirmed cases and 9 deaths, while in 2012, a total of 161 confirmed cases and 18 deaths were reported mainly in Attica and north-eastern Greece. A strain of lineage 2 was detected in 2010 in pools of Culex mosquitoes [35] and in wild birds [20]. In this study we correlate various environmental factors with WNV maintenance, amplification and potential for future spread in Greece.

Various public health studies have used Geographical Information System technologies as a tool for data analysis [2,9,15]. Previous studies [9,10] found that certain social and environmental factors were correlated with WNV dissemination patterns: The presence of vegetation, distance to a WNV positive dead bird, the intensity of mosquito abatement, demographic factors such as population age, race and financial status. Low precipitation and warm temperature were also found to associate with WNV cases. On the other hand, spread of WNV has shown some unique distribution patterns in different regions [15,30].

Before reaching the aforementioned results, we undertook several efforts to find out a pattern, or a distinguishable attribute of the WNV positive cases in Greece. Although we used a significant number of explanatory variables for describing each positive case (a mix of both continuous and categorical variables), there was no indication that these altogether could show the pattern in question. Therefore we tried to reduce this dataset by using Factor Analysis. We run PCA once for the temperature variables and once for the precipitation variables. Two components (93% of the variation was explained) for the temperature and two components (96% of the variation) for the precipitation variables were extracted, which means that the fit was very good. These four variables with the rest demographic and environmental variables were used in the Two Step Cluster method. This method was preferred compared to other clustering techniques because it can handle both categorical and continuous

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variables. However, we also run hierarchical cluster analysis using only the continuous variables, but no pattern of the positive human cases was revealed. Therefore, we used the two step cluster technique in a backward selection way. Initially we used all the explanatory variables together, and we removed one at a time if the Silhouette indicator was not considered good. We used the log-likelihood distance instead of Euclidean distance, because there were initially categorical variables in the dataset. However, when only the two continuous variables were left (‘‘altitude’’ and ‘‘distance from water’’), we checked also if the Euclidean distance could reveal the same pattern with the log- likelihood but it didn’t. We believe the fact that some of the variables were not significant for the clustering procedure was due to similar environmental conditions existing in Greece during summer. For example, there is no significant variation in terms of temperature or precipitation. This is why more stable variables like distance from water and altitude were responsible for the form of the clusters. After we formed the three clusters with these two variables, this pattern was revealed for both human cases and resident wild birds seroprevalence data.

Distance to water and altitude have both been previously shown to be negatively correlated with mosquito larval presence [36]; mosquitoes are the main biological vectors of WNV and transmission of this arthropod-borne virus is highly dependent on the density of mosquitoes. Low lying areas in close proximity to water include wetland habitats that are used as resting and breeding areas for various migratory and resident birds, allowing the long-distance introduction of the virus via migration routes as well as the rapid local amplification of the virus in a mosquito-bird cycle. In this study, apart from statistically identifying proximity to water and altitude as risk factors of spread of WNV in Greece, we were able to determine specific mean values for these habitat variables that allowed us to predict areas at high-risk for further disease incursion.

WNV positive birds are considered important environmental predictors of WNV human risk and are used in surveillance and risk assessment [2,9,37]. Whilst viremic birds are likely to represent the highest risk to humans, the viremic phase is extremely short, restricting data richness and thus statistical power. Hence we focused our analysis on longer lived serological measures of

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exposure. Moreover, the use of only the resident WNV seropositive wild birds from all hunter-harvested samples available, even though samples from migratory birds were also found positive, increased the reliability of our analysis, avoiding biases regarding area of exposure e.g. migratory birds travel long distances so the origin of exposure is hard to be determined. Hence, this is a good example of a case in which surveillance regarding exposure and other similar biological data derived from nature, regarding a zoonosis, can be used as an indicator for predicting high-risk areas. This fact was confirmed by the good fit that our model showed for the 2012 positive WNV human cases in Greece.

5.5 Conclusions

Modelling results indicated that positive resident wild bird occurrences are correlated with human WNV risk and can facilitate the assessment of environmental variables that contribute to that risk, recognizing new high-risk areas where the disease could further spread. Our approach allowed us to create a risk based mapping system to assist and guide WNV disease surveillance, monitoring and control. This risk based approach offers a way to stratify surveillance efforts and resources to improve the efficiency of surveillance for new outbreaks and monitoring existing outbreaks. Furthermore, it could proactively enhance other preventive efforts and educational campaigns for the general public in the not yet ‘‘affected’’ areas. Most importantly, early warning and identification of outbreaks is critical to limiting the animal and human losses to this disease. An active surveillance program undertaken on resident wild birds could be added to active and passive surveillance focused on humans, horses and mosquitoes greatly helping in evaluating and dealing with future outbreaks linked to flaviviruses.

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CHAPTER 6

APPLICATION OF MICROARRAYS TO WILD AVIAN TISSUE SAMPLES FOR SCREENING WEST NILE VIRUS AND OTHER AVIAN VIRUSES: TECHNICAL ASPECTS AND CONSIDERATIONS

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Summary

The use of multiplex diagnostic molecular technologies offers great advantages over conventional methods for the detection of presence or exposure to important pathogens. Simultaneous investigation of multiple pathogens renders these technologies attractive for diseases surveillance in avian species and especially wild ones, where small quantities of samples are usually available. Application of microarray technology for use in detecting simultaneously the exposure of wild birds to various viral pathogens is presented in this chapter. Tissue samples from hunter-harvested wild birds in Greece as well as selected archived wild avian tissue samples collected in Spain, were used in a general screening study using microarray technology under development. Using this novel technology we were able to detect the presence of genomic material of two viruses (West Nile Virus and Usutu virus) in two samples. These first results are encouraging regarding the application of microarrays in surveillance programs established by public health authorities. Perspectives and difficulties of using these assays as screening tools are discussed.

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6.1 Introduction

The use of multiplex diagnostic molecular technologies offers great advantages over conventional methods for the detection of presence or exposure to important pathogens. Simultaneous investigation of multiple pathogens renders these technologies attractive for diseases surveillance in avian species and especially wild ones, where small quantities of samples are usually available. Application of microarray technology for use in detecting simultaneously the exposure of wild birds to various viral pathogens is presented in this chapter. Tissue samples from hunter-harvested wild birds in Greece as well as selected archived wild avian tissue samples collected in Spain, were used in a general screening study using microarray technology under development. Perspectives and difficulties of using these assays as screening tools are discussed.

6.2 Materials and Methods

A multiplex avian mini DNA microarray was used for the investigation of viral agents in wild bird samples from Greece and Spain. This microarray was developed by Sonal et al (1) and consists of approximately 600 probes which were designed on conserved genomic regions of various important avian viruses (Table 1). The array is printed in an 8-well strip format (Array Strip Format®, Alere Technologies®) so that, on each strip, 8 arrays are available and thus 8 samples can be simultaneously tested.

Virus family	Virus groups	Probe region	Number of probes
Herpesviridae	Alphaherpesvirus	DNA polymerase	6
Astroviridae	Astrovirus	ORF1a	29
Poxviridae	Avipox virus	P4b gene	3
Bornaviridae	Borna disease virus	M gene	7
Circoviridae	Circovirus	Rep gene	65
	Gyrovirus	ORF1 (V1 protein)	3
Coronaviridae	Coronavirus	N gene	57
Togaviridae	Eastern equine encephalitis virus	E2 gene	30
Birnaviridae	Gumboro disease virus	VP1 gene	52
Orthomyxoviridae	Influenza A virus	M gene	41
Paramyxoviridae	Metapneumovirus	F gene	14
	Paramyxovirus 1-12	M and F gene	90

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Parvoviridae	Parvovirus	VP3 gene	12
Picornaviridae	Picornavirus	3D gene	44
	Duck Hepatitis A Virus		7
Polyomaviridae	Polyomavirus	VP1 and VP2 genes	8
Reoviridae	Reovirus	S1 gene	27
Togaviridae	Sindbis virus	NSP1	5
Flaviviridae	Flavivirus (other)	NS5	6
	Japanese encephalitis virus		5
	Murray Valley encephalitis virus		3
	St. Louis encephalitis virus		5
	Usutu virus		3
	West Nile virus		15
	Tick-borne encephalitis virus		20
	Louping ill virus		4
Adenoviridae	Adenovirus	Hexon	7
Hepeviridae	Avian Hepatitis E Virus	NS protein	3
Hepadnaviridae	Duck Hepatitis B Virus	S gene	4

Table 1. Genomic region and number of probes designed for each viral pathogen and printed on the avian mini DNA microarray.

The technology was applied on a total of 120 samples from Greece and Spain: The 89 Samples from Greece were of unknown infectious status, hunter-harvested during hunting seasons by members of Hunting Federations (Table 2). Using the microarray technology, these samples were simultaneously screened for pathogens of interest for the purposes of three projects: various wild avian viruses for the WildTech FP7 Project, West Nile virus for the MALWEST Project and enterotropic viruses of zoonotic potential for a THALES Project. The 31 Spanish samples were of known infectious status and origin but only to the sender of the samples (blind study).

N	Bird Species	Area of Collection	Hunting Season	Tissue
1	*Anas crecca*	Thessaloniki	2012-2013	Heart
2	*Anas crecca*	Thessaloniki	2012-2013	Heart
3	*Anas crecca*	Thessaloniki	2012-2013	Heart
4	*Anas crecca*	Thessaloniki	2012-2013	Heart
5	*Anas crecca*	Thessaloniki	2012-2013	Heart

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6	*Anas crecca*	Thessaloniki	2012-2013	Heart
7	*Anas crecca*	Thessaloniki	2012-2013	Heart
8	*Anas platyrhynchos*	Aksios River	2012-2013	Heart, Liver
9	*Anas platyrhynchos*	Aksios River	2012-2013	Heart, Liver, Brain
10	*Anas platyrhynchos*	Aksios River	2012-2013	Heart, Liver
11	*Anas platyrhynchos*	Aksios River	2012-2013	Heart, Liver
12	*Podiceps cristatus*	Volvi Lake	2012-2013	Liver
13	*Aythya ferina*	Volvi Lake	2012-2013	Liver
14	*Podiceps cristatus*	Volvi Lake	2011-2012	Liver
15	*Anas platyrhynchos*	Thessaloniki	2011-2012	Liver, Pancreas
16	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
17	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
18	*Anas platyrhynchos*	Ksanthi	2012-2013	Heart, Liver
19	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
20	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
21	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
22	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
23	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
24	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
25	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
26	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
27	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
28	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
29	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
30	*Anas crecca*	Ksanthi	2012-2013	Liver
31	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
32	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
33	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
34	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
35	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
36	*Anas crecca*	Ksanthi	2012-2013	Heart, Liver
37	*Passer domesticus*	Karditsa	2013-2014	Heart, Liver, Pancreas
38	*Passer domesticus*	Athens	2013-2014	Heart, Pancreas
39	*Carduelis carduelis*	Attica	2013-2014	Heart, Liver, Pancreas

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40	*Acrocephalus sp*	Viotia	2013-2014	Heart, Liver, Pancreas
41	*Hirundo rustica*	Attica	2013-2014	Heart, Pancreas
42	*Hirundo rustica*	Atalanti	2013-2014	Liver
43	*Passer domesticus*	Attica	2013-2014	Liver
44	*Turdus merula*	Attica	2013-2014	Heart, Liver, Pacreas, Spleen
45	*Pica pica*	Farsala	2013-2014	Heart, Liver
46	*Anas penelope*	Ksanthi	2012-2013	Heart, Liver
47	*Anas penelope*	Ksanthi	2012-2013	Heart, Liver
48	*Scolopax rusticola*	Ksanthi	2012-2013	Heart, Liver
49	*Anas platyrhynchos*	Chalastra	2013-2014	Liver
50	*Anas platyrhynchos*	Chalastra	2013-2014	Liver
51	*Anas crecca*	Chalastra	2013-2014	Liver
52	*Anas platyrhynchos*	Chalastra	2013-2014	Liver
53	*Anas crecca*	Chalastra	2012-2013	Liver
54	*Anas crecca*	Chalastra	2012-2013	Liver
55	*Anas crecca*	Chalastra	2012-2013	Liver
56	*Anas crecca*	Chalastra	2012-2013	Liver
57	*Anas crecca*	Chalastra	2012-2013	Liver
58	*Pica pica*	Markopoulo	2013-2014	Liver
59	*Pica pica*	Markopoulo	2013-2014	Liver
60	*Pica pica*	Markopoulo	2013-2014	Liver
61	*Pica pica*	Markopoulo	2013-2014	Liver
62	*Pica pica*	Markopoulo	2013-2014	Liver
63	*Pica pica*	Spata	2013-2014	Liver
64	*Pica pica*	Spata	2013-2014	Liver
65	*Pica pica*	Spata	2013-2014	Liver
66	*Gallinago gallinago*	Evros	2012-2013	Liver
67	*Anas penelope*	Chalastra	2012-2013	Liver
68	*Anas penelope*	Chalastra	2012-2013	Liver
69	*Anas acuta*	Chalastra	2012-2013	Liver
70	*Anas querquedula*	Chalastra	2012-2013	Liver

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71	*Anas acuta*	Chalastra	2012-2013	Liver
72	*Anas penelope*	Chalastra	2012-2013	Liver
73	*Anas crecca*	Chalastra	2011-2012	Liver
74	*Anas crecca*	Chalastra	2011-2012	Liver
75	*Anas crecca*	Chalastra	2011-2012	Liver
76	*Anas crecca*	Chalastra	2011-2012	Liver
77	*Anas crecca*	Chalastra	2011-2012	Liver
78	*Anas crecca*	Chalastra	2011-2012	Liver
79	*Anas crecca*	Chalastra	2011-2012	Liver
80	*Scolopax rusticola*	Vasilika	2011-2012	Liver
81	*Columba livia*	Serres	2012-2013	Liver
82	*Columba livia*	Serres	2012-2013	Heart
83	*Anas platyrhynchos*	Serres	2012-2013	Liver
84	*Anas platyrhynchos*	Serres	2012-2013	Liver
85	*Anas platyrhynchos*	Serres	2012-2013	Liver
86	*Tadorna tadorna*	Serres	2012-2013	Liver
87	*Anas stepera*	Serres	2012-2013	Liver
88	*Anas platyrhynchos*	Serres	2012-2013	Liver
89	*Anas platyrhynchos*	Serres	2012-2013	Liver

Table 2. Avian Samples derived from Greece. These samples were hunter-harvested by members of the Greek Hunting Federations during the hunting seasons.

For the sample preparation, a quantity of 50 mg of the tissue sample is homogenized using 750 μl Trizol and sterilized glass beads, with continuous vortexing. A quantity of 250 μl chloroform is added, and after vortexing for 1 minute, the sample is centrifuged for 15000 rpm for 15 minutes at a temperature of $4^{o}C$. A total of 300 μl of the top aqueous solution is removed and placed in a special tube used with the EZ1 Advanced XL instrument (Qiagen®) of automated nucleic acid extraction. Extraction is performed according to manufacturer instructions and quantity/quality of genomic extract is checked using Nanodrop (Thermoscientific®). DNA and RNA concentration is then adjusted to 200 μg/μl. Total quantities needed are 11 μl of DNA solution and 8 μl RNA solution.

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DNA is digested in the RNA solution using DNAse: 8 μl RNA solution + 1 DNAse I (Invitrogen®) + 1 μl 10X Dnase buffer (Invitrogen®). After incubation for 30 minutes at 37°C, 1 μl of 25mM EDTA is added and incubated at 65°C for 10 minutes.

A unique combination of random amplification and specific biotin labelling is performed, adapting procedures used by Gurrala et al., 2009 (2). Reverse transcription and Polymerase Chain Reaction (PCR) is performed using Random primer A as follows:

Step 1 - For the RNA solution: 1μl Random Primer A (40 μM) + 1 μl mM dNTP Mix + 11 μl RNA Template. Heat at 95°C for 5 minutes and immediately put on ice.

Step 2 - For the RNA solution: Add 4 μl 5X RT buffer + 1 μl 0.1 DTT + 1 μl RNase inhibitor + 1 μl Superscript III in the previous mix. For the DNA solution: Add 4 μl 5X RT buffer + 1 μl 0.1 DTT + 2 μl water in the 11 μl DNA solution. Incubate both mixes at 25°C for 5 minutes, 42°C for 50 minutes, and 70°C for 15 minutes.

Step 3 - Incubate both mixes at 94°C for 2 minutes, cool at 10°C. Add 7,7 μl water, 2 μl 5X Sequenase buffer and 0,3 μl Sequenase. Heat at 37°C for 8 minutes, at 94°C for 2 minutes and cool at 10°C.

PCR using Primer B (complementary to first part of random Primer A) is then performed as follows: A PCR mix consisting of 37,5 μl water, 5 μl 10X KlenTaq PCR buffer (Sigma®), 1 μl 12,5 mM dNTP, 1 μl 100 μM primer B, 0,5 μl KlenTaq LA polymerase (5u/μl) and 5 μl dsDNA (2,5 μl cDNA and 2,5 μl DNA) is created. PCR conditions: 94 °C for 4 minutes, 68 °C for 5 minutes, 35 cycles of 94 °C for 30 seconds, 50 °C for 1 minute, 68 °C for 1 minute. Finally 68 °C for 2 minutes and cool at 10 °C (Figure 1).

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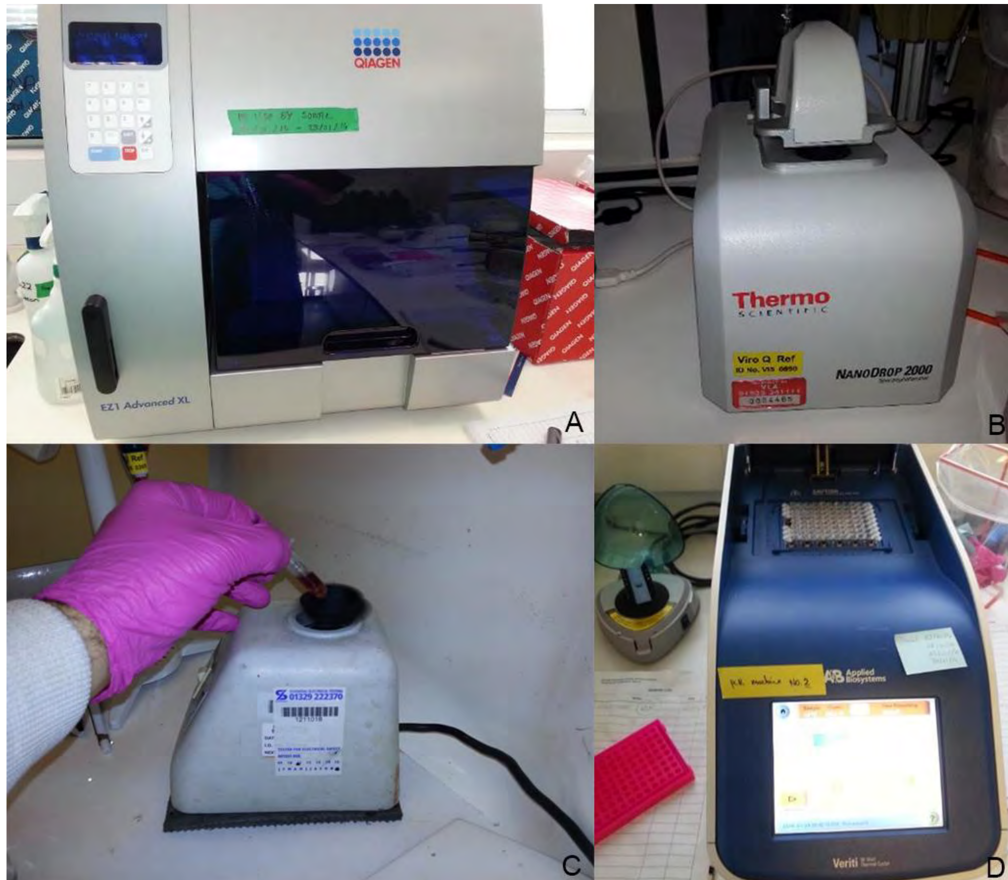

Figure 1. Equipment used during sample preparation and PCR. A) EZ1 Advanced XL instrument (Qiagen®) B) Nanodrop (Thermoscientific®) C) Vortex D) PCR Thermocycler (Applied Biosystems®).

Gel electrophoresis of the PCR product is performed after this step to observe the presence of DNA and cDNA of 300-1000 bp size (Figure 2).

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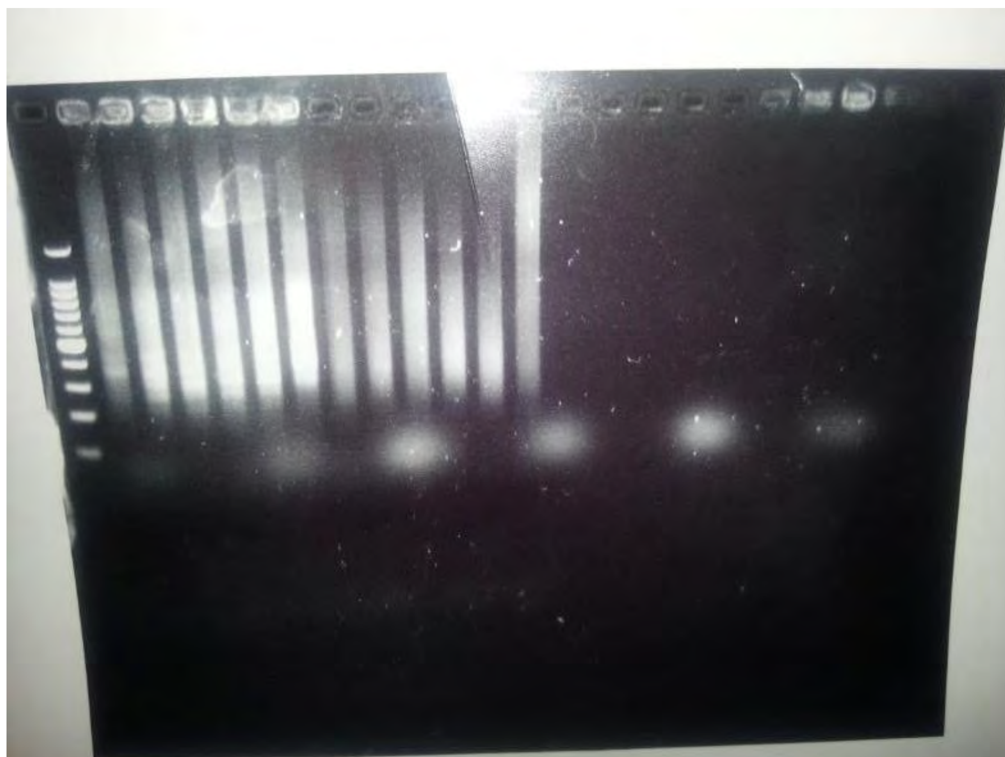

Figure 2. Picture of gel after electrophoresis where amplicons of appropriate size (300-1000 bp) are detected. A DNA Ladder of 100 bp size bands is used (except top band which is of 1500 bp size).

Labelling PCR is finally performed to label amplicons with biotin. 5 μl of PCR product of Primer B PCR + 1 μl 10X KlenTaq PCR buffer + 1 μl dNTP mix + 2,45 μl mix of specific Primers + 0,2 μl KlenTaq LA polymerase (5 u/μl) + 0,35 μl Biotin -16-dUTP. PCR conditions: 94 °C for 4 minutes, 68 °C for 5 minutes. 25 cycles at 94 °C for 30 seconds, 60 °C for 1 minute, 50 °C for 1 minute, 68 °C for 1 minute. Finally 68 °C for 2 minutes and cool at 10 °C.

Application of the microarray technology was performed using the Identibac hybridization kit (Alere Technologies®) according to the manufacturer instructions. The protocol is as follows:

- Preparation of samples: 10 μl of labelled sample + 90 μl of hybridization buffer C1. Heat at 95°C and immediately cool on ice.
- Microarray preparation: Well washing with 200 μl PCR-water (Pipetting 4 times in room temperature). Add of 200 μl buffer C1. Heat at 60°C for 2 minutes at 550 rpm.

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- Hybridization: Add 100 μl of sample. Incubate at 60°C for 60 minutes at 550 rpm.
- Washing: First wash with 200 μl washing buffer C2. Remove without incubation. Second wash with 200 μl washing buffer C2. Incubate at 60°C for 5 minutes at 550 rpm. Third wash with 200 μl washing buffer C2. Incubate at 60°C for 5 minutes at 550 rpm.
- Addition of Conjugate: Add 100 μl C3:C4 (1X HRP-Streptavidin Conjugate Solution). Incubate at 30°C for 10 minutes at 550 rpm.
- Washing: Add 200 μl washing buffer C5. Pipetting 2 times at room temperature. Repeat once.
- Addition of Substrate: Add 100 μl D1 TMB substrate. Incubate 25°C for 10 minutes.

If a specific viral amplicon is present in the sample, the amplicon will be attached on the complementary probes on specific spots of the microarray. As the amplicon has been biotinylated, the streptavidin-HRP conjugate will be attached and will cause the change of the colour of the TMB substrate on the specific spot. Signal is produced by the change of colour of the TMB substrate, on the spots that labelled amplicons have been attached. The signal of the intensity of the colour is read at the Arraymate Platform (Alere Technologies®) and data is extracted in an excel spreadsheet (Figure 2).

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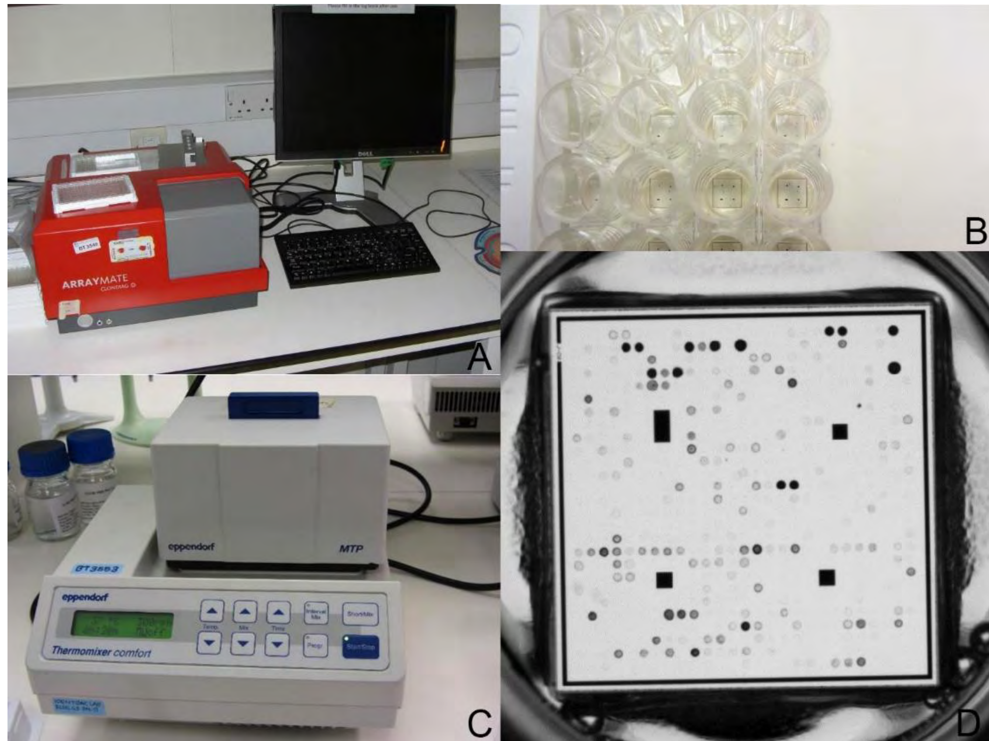

Figure 2. Application of Arraystrip Microarray Technology A) ArrayMate Platform (Alere Technologies®) B) ArrayStrip Format (Alere Technologies®) C) Thermomixer (Eppendorf®) D) Example of Image of Array after procedure as taken by the ArrayMate.

The raw data is being processed by an algorithm that groups the signal of spots per pathogens and normalizes them (reducing background noise) (Figure 3). When an increased signal is recognized on spots of a specific viral pathogen, confirmation PCR using established protocols is performed to verify the result.

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	Probes	meanDif	medianDif	sdError
Usutu Virus	3	0.602605	0.613194	80%-90%
Murray Valley Encephalitis Virus	3	0.5726995	0.85509	>90%
Biotin-Marke	1	0.43604425	0.43604425	NA
0,1M NaPP Standard pH 9	1	-0.21182525	-0.21182525	NA
Sindbis Virus	5	0.0987624	0.0021235	80%-90%
Coronavirus	57	0.089151298245614	0.0150585	<75%
Polyomavirus	8	0.0763849375	0.0057845	80%-90%
Circovirus	52	0.0687192788461539	0.0002365	<75%
Bourna Disease Virus	7	-0.0664938571428571	-0.0260435	<75%
Chicken Anemia Virus	13	0.0661477307692308	0.0063085	75%-80%
Reovirus	27	-0.0620699444444444	-0.01275	<75%
TBE	20	-0.057739725	-0.01246325	80%-90%
HybControl-1	1	-0.053643	-0.053643	NA
St. Louis Encephalitis Virus	5	0.0508958	-0.0131815	80%-90%
Isreal Turkey	2	-0.04728525	-0.04728525	75%-80%
HepA	7	0.0465524285714286	0.017295	<75%
West Nile Virus	15	0.0313575333333333	0.0073355	<75%
Alpha Herpes	6	-0.0288071666666667	-0.018128	<75%
Adenovirus	7	-0.0244227142857143	-0.0090985	<75%
Control	7	0.0237566428571429	0.008451	<75%
aMPV	104	0.0174083125	0.00055425	<75%
DuckHaem_flavi	2	0.01667675	0.01667675	<75%
Tembusu	1	0.015187	0.015187	NA
Japanese Encephalitis Virus	5	-0.0132202	0.0030265	<75%
Avipoxvirus	3	-0.00797633333333333	-0.0020075	<75%
LIV	4	-0.0069175	-0.0071325	<75%
Flavivirus Duck	1	0.00336	0.00336	NA
Picornavirus	43	0.00273598837209302	-0.001617	<75%
Infectious Bursal Disease	52	-0.00252404807692308	-0.001672	<75%
HepB&E	7	-0.00202871428571429	-0.0005115	<75%
Dependovirus	13	0.00177742307692308	-0.0013815	<75%
Influenza A Neg	41	0.00157703658536585	-0.001294	<75%
Gyrovirus	3	-0.000610500000000001	-4.05e-05	<75%
Avastrovirus	29	0.000232068965517247	-0.0071265	<75%
Eastern Equine Encephalitis Virus	30	-0.000179866666666666	0.00044475	<75%

Figure 3. Example of Signal results after data analysis. Signals of Usutu and Murray Valley Encephalitis Virus are considered high (maximum signal value of 1.0) and appropriate for application of confirmation PCR.

6.3 Results

During the screening of the 120 samples, a total of 13 samples were considered suspect for the presence of viral pathogens (Table 3).

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Sample ID	Bird Species	Viral Pathogen Suspect
1	*Anas crecca*	Sindbis Virus
2	*Anas crecca*	Sindbis Virus
9	*Anas platyrhynchos*	Hepatitis A Virus
10	*Anas platyrhynchos*	West Nile Virus
18	*Anas platyrhynchos*	Sindbis Virus
24	*Anas crecca*	Flaviviruses
32	*Anas crecca*	Flaviviruses
49	*Anas platyrhynchos*	Hepatitis A Virus
64	*Pica pica*	West Nile virus
86	*Tadorna tadorna*	Avian poxvirus
88	*Anas platyrhynchos*	Sindbis Virus
89	*Anas platyrhynchos*	Sindbis Virus
S21	*Turdus philomelos*	Usutu Virus

Table 3. Samples recognized as suspect for the presence of viral pathogens by the avian mini DNA microarray platform.

During confirmation PCR, two samples were being confirmed: Sample no 24 for the presence of West Nile virus RNA and sample S21 for the presence of Usutu Virus. For the Spanish sample, it was confirmed that it was a song thrush infected with Usutu virus, as was already reported (3).

6.4 Discussion

During this study, application of molecular microarray platform to wild avian samples was performed to evaluate the possibility of using this technology as a screening tool to samples of unknown status. Using a screening tool like this could allow the timely detection of viral pathogens introduced by wild birds in an area. Using this novel technology we were able to detect the presence of genomic material of two viruses (West Nile Virus and Usutu virus) in two samples. These first results are encouraging regarding the application of microarrays in surveillance programs established by public health authorities. Procedure is cost effective and can give fast preliminary results, focusing surveillance effort in specific targets detected by this first multiplex screening format.

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However, this technology has also some major technical considerations. Development of a microarray format needs a significant amount of preliminary work regarding specific probes design for each pathogen: Probes must be harmonized regarding parameters of melting temperature, G+C content, and self-annealing capacity, avoiding at the same time cross-hybridizations. Moreover, ordering the arrays in strip format must be done in bulk by the producing companies, demanding a first large financial investment, something that can be performed only by large-sized laboratories.

Data analysis is also demanding: It is almost impossible to define specific cut-offs regarding the signal intensity that would allow the discrimination between negative and positive samples. Samples may only be defined as "suspect" using this technology that should be further confirmed by established molecular methods. In our case, 2 of the 13 suspect samples were confirmed by PCR. Of course, questions arise regarding the possibility that this technology has an increased sensitivity in comparison to the known established detection methods. However, this is something very difficult to be tested, and only in a probe-specific and pathogen-specific aspect.

Application of microarray technologies for screening purposes on wild avian samples is promising. This technology can be very useful for surveillance programs and could also allow the early identification of the causative agent in an initial outbreak of a disease of unknown aetiology. For example in the case of the West Nile virus outbreak in Greece, screening of wild avian samples in a routine pattern by the public health authorities could possibly allow the detection of the pathogen many months earlier than the human disease outbreak. This would give the opportunity to the relevant authorities to apply prevention measures that could minimize the consequences of the outbreak and protect more effectively Public Health.

6.5 References

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GENERAL CONCLUSIONS

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GENERAL CONCLUSIONS

Studies included in the current PhD thesis led to some important findings:

- A WNV strain sequence derived from a Eurasian magpie hunted during the outbreak of the human disease in Greece (2010-2014) was found and reported. This was the first report of WNV RNA detection in wild birds in the country. The sequence has highest sequence similarity to Lineage 2 strain sequences from birds of prey in Austria obtained in 2008 and 2009. This finding implicates this corvid species in a local virus maintenance and generates concerns about possible overwintering and expansion of the virus in neighbouring areas.
- First serological results of wild avian samples showed association of human cases with wild birds' exposure to the virus; none of the sera derived from birds hunter-harvested in prefectures unaffected by the WNV human outbreak was found positive. On the contrary, birds were found to be exposed to WNV in every prefecture that reported human cases and from which avian sera were available ($\chi^2 = 12.5$, df = 1, $P < 0.001$). High seroprevalence of resident wild birds (Eurasian magpies) suggested a high activity of WNV in the affected areas.
- WNV-reactive antibodies were detected in sera of migratory turtle doves which were hunter-harvested on the day of their arrival at resting areas of Greece during their autumn migration from Central Europe to wintering areas of Africa. This demonstrates past exposure to WNV probably to the areas of their origin. The high molecular similarity of the WNV magpie strain detected in Greece with strains derived from Austria and Hungary suggests a southwards dispersion of WNV from Central Europe which may have been caused through the migration routes of migratory avian species with similar migration traits (turtle doves, quails, orioles, etc.)
- Resident corvids hunter-harvested in the epicentre of the outbreak have been exposed to WNV at least eight months before the first human cases were reported. Thus an active wildlife surveillance system for emerging infectious diseases would predict the mosquito-wild birds WNV circulation and the possible emergence under appropriate conditions that caused the major outbreak in humans.

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- Genetic determinants of increased virulence were present in the WNV strain detected that further support the implication of wild birds in the epidemiology of the disease.
- A monitoring system was established with wild birds and reported human cases data using Geographical Information System (GIS). Potential distribution of WNV was modelled by combining wild bird serological surveillance data with environmental factors (e.g. elevation, slope, land use, vegetation density, temperature, precipitation indices, and population density). Using GIS analysis, the identified risk factors were applied across Greece identifying new high risk-areas for further disease spread. The results of the analysis were evaluated and confirmed using the 161 reported human cases of the 2012 outbreak predicting correctly (Odds = 130/31 = 4,194 95%CI = 2,841–6,189) and more areas were identified for potential dispersion in the following years. This approach verified that WNV risk can be modelled in a fast cost-effective way indicating high risk areas where prevention measures should be implemented in order to reduce the disease incidence.
- Application of a molecular microarray platform to wild avian samples was performed to evaluate the possibility of using this technology as a screening tool to samples of unknown status. Using a screening tool like this could allow the timely detection of viral pathogens introduced by wild birds in an area. Using this novel technology we were able to detect the presence of genomic material of two viruses (West Nile Virus and Usutu virus) in two samples, demonstrating the potential of applying these multiplex techniques as screening tools in samples of unknown infectious status by public health authorities in a routine surveillance scheme.

All these findings demonstrate the direct and indirect implication of wild birds in the recent WNV outbreak in Greece and the importance of correlating wildlife and human cases data in application of screening and data analysis tools. Results add value to the need of a One Health approach in establishing monitoring and prevention programs regarding zoonoses. West Nile virus is an excellent example of the interplay of driving forces of pathogen exchange between wild animals, domestic animals and humans. This thesis findings support the view that multidisciplinary co-operation of medical and veterinary

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authorities and institutes is necessary for monitoring and collecting/analysing data needed for applying the appropriate disease control and prevention measures, protecting in the most successful way Public and Animal Health.

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UNIVERSITY OF THESSALY
SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE
DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

WILD BIRDS AS A SOURCE OF SELECTED EMERGING AND RE-EMERGING PATHOGENS: THE WEST NILE VIRUS EXAMPLE IN GREECE

Doctoral Thesis by
Valiakos Georgios

Summary

Since 2010, a major West Nile virus (WNV) outbreak of human infections took place in Greece. From 2010 until 2014 more than 624 laboratory-confirmed cases and 73 deaths were caused by the virus. This thesis reports several studies conducted to investigate possible implication of wild birds during the WNV outbreak in Greece. Moreover, this thesis reports studies conducted to evaluate the application of novel technologies and tools like Geographical Information Systems (GIS) and multiplex diagnostic techniques (microarrays) in wild birds surveillance programs in terms of stratifying surveillance efforts and resources and improving the efficiency of monitoring existing outbreaks and timely recognizing new ones.

A WNV lineage 2 strain was molecularly identified and characterised in a Eurasian magpie hunted in Greece in 2010, during a WNV outbreak in humans. This is the first report of detection of WNV RNA in wild birds, and the first direct implication of wild birds to the outbreak. Phylogenetic analysis revealed the highest sequence similarity (>99%) with other WNV lineage 2 strains derived from birds of prey in Austria and Hungary (2004–2009). A serological and molecular surveillance was further conducted on samples of hunter-harvested wild birds prior to and during the outbreak, as well as on samples derived from various areas of mainland Greece.

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Fourteen positive sera were obtained from birds hunter-harvested up to 8 months prior to the human outbreak. Specific genetic determinants of virulence (His249Pro NS3 mutation, E-glycosylation motif) were recognized in the WNV lineage 2 magpie strain and a nucleotide mismatch was revealed between this strain and a mosquito WNV strain isolated one month earlier in the same area. This is the first report regarding exposure of wild birds to WNV prior to the 2010 outbreak, in Greece. Results provide evidence of the implication of wild birds in a local enzootic cycle that could allow maintenance and amplification of the virus before and during the outbreak. Findings of past exposure of migratory birds to WNV upon their arrival in Greece during autumn migration, suggest avian species with similar migration traits as candidates for the introduction of WNV into Greece. Results showed association of human cases with wild birds' exposure to the virus; no avian sera were found positive in prefectures not affected by the WNV outbreak. In contrast, positive avian sera were found in every prefecture that human WNV cases occurred in 2011.

Another aim of this thesis was to establish a monitoring system with wild birds and reported human cases data using Geographical Information System (GIS). Potential distribution of WNV was modelled by combining wild bird serological surveillance data with environmental factors (e.g. elevation, slope, land use, vegetation density, temperature, precipitation indices, and population density). Local factors including areas of low altitude and proximity to water were important predictors of appearance of both human and wild bird cases (Odds Ratio = 1,001 95%CI = 0,723–1,386). Using GIS analysis, the identified risk factors were applied across Greece identifying the northern part of Greece (Macedonia, Thrace) western Greece and a number of Greek islands as being at highest risk of future outbreaks. The results of the analysis were evaluated and confirmed using the 161 reported human cases of the 2012 outbreak predicting correctly (Odds = 130/31 = 4,194 95%CI = 2,841–6,189) and more areas were identified for potential dispersion in the following years. Our approach verified that WNV risk can be modelled in a fast cost-effective way indicating high risk areas where prevention measures should be implemented in order to reduce the disease incidence.

Lastly, tissue samples from hunter-harvested wild birds in Greece as well as selected archived wild avian tissue samples collected in Spain, were used in a general screening study using microarray technology under development. Using this novel

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technology we were able to detect the presence of genomic material of two viruses (West Nile Virus and Usutu virus) in two samples. These first results are encouraging regarding the application of microarrays in surveillance programs established by public health authorities. Perspectives and difficulties of using these assays as screening tools are discussed.

All these findings demonstrate the direct and indirect implication of wild birds in the recent WNV outbreak in Greece and the importance of correlating wildlife and human cases data in application of screening and data analysis tools. Results add value to the need of a One Health approach in establishing monitoring and prevention programs regarding zoonoses. West Nile virus is an excellent example of the interplay of driving forces of pathogen exchange between wild animals, domestic animals and humans. This thesis findings support the view that multidisciplinary co-operation of medical and veterinary authorities and institutes is necessary for monitoring and collecting/analysing data needed for applying the appropriate disease control and prevention measures, protecting in the most successful way Public and Animal Health.

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ
ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΠΑΡΑΣΙΤΟΛΟΓΙΑΣ

ΤΑ ΑΓΡΙΑ ΠΤΗΝΑ ΩΣ ΦΟΡΕΙΣ ΕΠΙΛΕΓΜΕΝΩΝ ΑΝΑΔΥΟΜΕΝΩΝ ΚΑΙ ΕΠΑΝΑΔΥΟΜΕΝΩΝ ΠΑΘΟΓΟΝΩΝ: ΤΟ ΠΑΡΑΔΕΙΓΜΑ ΤΟΥ ΙΟΥ ΤΟΥ ΔΥΤΙΚΟΥ ΝΕΙΛΟΥ ΣΤΗΝ ΕΛΛΑΔΑ

Διδακτορική Διατριβή του
Γεωργίου Βαλιάκου

Περίληψη

Από το 2010, μία σημαντικά έξαρση ανθρώπινων κρουσμάτων της νόσου του Ιού του Δυτικού Νείλου (ΙΔΝ) έλαβε χώρα στην Ελλάδα. Από το 2010 μέχρι το 2014 πάνω από 624 εργαστηριακά επιβεβαιωμένα κρούσματα και 73 θάνατοι προκλήθηκαν από τον ιό. Η παρούσα διατριβή αναφέρει αρκετές μελέτες που πραγματοποιήθηκαν για να διερευνηθεί πιθανή εμπλοκή των άγριων πτηνών κατά τη διάρκεια της επιδημίας του ΙΔΝ στην Ελλάδα. Επιπλέον, αυτή η διατριβή αναφέρει μελέτες που διεξήχθησαν για την αξιολόγηση της εφαρμογής νέων τεχνολογιών και εργαλείων όπως Γεωγραφικά Συστήματα Πληροφοριών (GIS) και multiplex διαγνωστικές τεχνικές (microarrays) σε προγράμματα επιτήρησης άγριων πτηνών με σκοπό την εξοικονόμηση των προσπαθειών επιτήρησης και των πόρων και τη βελτίωση της αποτελεσματικότητας της παρακολούθησης των υφιστάμενων εστιών και έγκαιρη αναγνώριση νέων.

Ένα στέλεχος του ιού του τύπου 2 αναγνωρίστηκε μοριακά και χαρακτηρίστηκε σε μια καρακάξα η οποία και θηρεύτηκε στην Ελλάδα το 2010, κατά τη διάρκεια μιας επιδημίας του ΙΔΝ σε ανθρώπους. Αυτή είναι η πρώτη αναφορά ανίχνευσης RNA του ΙΔΝ σε άγρια πτηνά, και η πρώτη άμεση εμπλοκή των άγριων πτηνών στην έξαρση. Η φυλογενετική ανάλυση έδειξε την μεγαλύτερη ομοιότητα ακολουθίας (> 99%) με άλλα

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στελέχη του ΙΔΝ που προέρχονται από αρπακτικά πουλιά της Αυστρίας και της Ουγγαρίας (2004-2009). Η ορολογική και μοριακή επιτήρηση επεκτάθηκε περαιτέρω σε δείγματα θηρευμένων αγρίων πτηνών πριν και κατά τη διάρκεια της επιδημίας, καθώς και σε δείγματα που προέρχονται από διάφορες περιοχές της ηπειρωτικής Ελλάδας.

Δεκατέσσερις θετικοί οροί ελήφθησαν από πτηνά τα οποία και θηρεύτηκαν μέχρι και 8 μήνες πριν από την έξαρση των ανθρώπινων κρουσμάτων. Ειδικοί γενετικοί παράγοντες παθογένειας (His249Pro NS3 μετάλλαξη, μοτίβο e-γλυκοσυλίωσης) αναγνωρίστηκαν στο στέλεχος του ιού που εντοπίστηκε στη καρακάξα και μία αναντιστοιχία νουκλεοτιδίων αποκαλύφθηκε μεταξύ αυτού του στελέχους και ενός στελέχους που απομονώθηκε από κουνούπι ένα μήνα νωρίτερα στην ίδια περιοχή. Αυτή είναι η πρώτη αναφορά όσον αφορά την έκθεση των αγρίων πτηνών στον ΙΔΝ πριν από το ξέσπασμα του 2010, στην Ελλάδα. Τα αποτελέσματα προσφέρουν αποδείξεις σχετικά με την εμπλοκή των αγρίων πτηνών σε ένα τοπικό ενζωοτικό κύκλο που θα μπορούσε να επιτρέψει τη συντήρηση και ενίσχυση του ιού πριν και κατά τη διάρκεια της επιδημίας. Ευρήματα έκθεσης αποδημητικών πτηνών στον ΙΔΝ κατά την άφιξή τους στην Ελλάδα κατά τη φθινοπωρινή μετανάστευση, προτείνουν είδη πτηνών με παρόμοια μεταναστευτικά χαρακτηριστικά ως υποψήφια για την εισαγωγή του ΙΔΝ στην Ελλάδα. Τα αποτελέσματα έδειξαν συσχέτιση των ανθρώπινων κρουσμάτων με την έκθεση άγριων πτηνών στον ιό καθώς οροί πτηνών δε βρέθηκαν θετικοί σε νομούς που δεν επηρεάζονται από την επιδημία του ιού του ΔΝ. Αντίθετα, θετικοί οροί πτηνών βρέθηκαν σε κάθε νομό όπου αναφέρθηκαν ανθρώπινα κρούσματα της νόσου μέσα στο 2011.

Ένας άλλος στόχος της παρούσας διατριβής ήταν η καθιέρωση ενός συστήματος επιτήρησης με δεδομένα άγριων πτηνών και ανθρώπινων κρουσμάτων, με τη χρήση του Συστήματος Γεωγραφικών Πληροφοριών (GIS). Η πιθανή κατανομή του ΙΔΝ εκτιμήθηκε συνδυάζοντας τα δεδομένα ορολογικής επιτήρησης των άγριων πτηνών με περιβαλλοντικούς παράγοντες (π.χ. υψόμετρο, κλίση, χρήση γης, την πυκνότητα της βλάστησης, η θερμοκρασία, δείκτες καθίζηση, και η πυκνότητα του πληθυσμού). Τοπικοί παράγοντες συμπεριλαμβανομένων των περιοχών με χαμηλό υψόμετρο και η εγγύτητα με το νερό ήταν σημαντικοί παράγοντες πρόβλεψης της εμφάνισης του ιού (λόγος πιθανοτήτων = 1,001 95%CI = 0,723-1,386). Χρησιμοποιώντας την ανάλυση GIS, οι εντοπισμένοι παράγοντες κινδύνου εφαρμόζονται σε όλη την Ελλάδα που

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προσδιορίζουν το βόρειο τμήμα της Ελλάδα (Μακεδονία, Θράκη), τη Δυτική Ελλάδα και μια σειρά από ελληνικά νησιά, όπως είναι σε υψηλότερο κίνδυνο μελλοντικών κρουσμάτων. Τα αποτελέσματα της ανάλυσης αξιολογήθηκαν και επιβεβαιώθηκαν με τη χρήση των 161 ανθρώπινων κρουσμάτων της επιδημίας του 2012 προβλέποντας σωστά (Αποδόσεις = 130/31 = 4,194 95%CI = 2,841-6,189) και παράλληλα περισσότερες περιοχές εντοπίστηκαν ως υψηλού κινδύνου για τη πιθανή διασπορά κατά τα επόμενα έτη. Η προσέγγισή μας επαλήθευσε ότι ο κίνδυνος έκθεσης στον ΙΔΝ μπορεί να απεικονιστεί με ένα γρήγορο και οικονομικά αποδοτικό τρόπο, ο οποίος δείχνει τις περιοχές υψηλού κινδύνου, όπου θα πρέπει να εφαρμοστούν μέτρα πρόληψης, προκειμένου να μειωθεί η συχνότητα εμφάνισης της νόσου.

Τέλος, δείγματα ιστού από θηρευμένα άγρια πτηνά στην Ελλάδα, καθώς και επιλεγμένα αρχειακά δείγματα ιστών άγριων πτηνών που συλλέχθηκαν στην Ισπανία, χρησιμοποιήθηκαν σε μια γενική μελέτη διαλογής χρησιμοποιώντας τεχνολογία μικροσυστοιχιών υπό ανάπτυξη. Χρησιμοποιώντας αυτήν την νέα τεχνολογία ήμασταν σε θέση να ανιχνεύσει την παρουσία του γονιδιωματικού υλικού του δύο ιών (ΙΔΝ και ο ιός Usutu) σε δύο δείγματα. Αυτά τα πρώτα αποτελέσματα είναι ενθαρρυντικά όσον αφορά την εφαρμογή των μικροσυστοιχιών σε προγράμματα επιτήρησης που έχουν καθοριστεί από τις αρχές δημόσιας υγείας. Οι προοπτικές και οι δυσκολίες από τη χρήση αυτών των τεχνικών ως εργαλεία διαλογής συζητούνται.

Όλα αυτά τα ευρήματα καταδεικνύουν την άμεση και έμμεση επίπτωση των άγριων πτηνών κατά την πρόσφατη επιδημία του ιού του ΔΝ στην Ελλάδα και τη σημασία του συσχετισμού δεδομένων προερχόμενων από την άγρια πανίδα με προερχόμενα από ανθρώπινα κρούσματα κατά την εφαρμογή εργαλείων διαλογής και ανάλυσης. Τα αποτελέσματα προσθέτουν αξία στην ανάγκη μιας One Health προσέγγισης για τη θέσπιση προγραμμάτων παρακολούθησης και πρόληψης όσον αφορά τις ζωοανθρωπονόσους. Ο Ιός του Δυτικού Νείλου είναι ένα εξαιρετικό παράδειγμα της αλληλεπίδρασης των κινητήριων δυνάμεων της ανταλλαγής παθογόνων μεταξύ άγριων ζώων, κατοικίδιων και ανθρώπων. Τα ευρήματα της παρούσας διατριβής ενισχύουν την άποψη ότι η διεπιστημονική συνεργασία των ιατρικών και κτηνιατρικών αρχών και ιδρυμάτων είναι απαραίτητη για την παρακολούθηση και τη συλλογή/ανάλυση των δεδομένων που απαιτείται ώστε να μπορούν να εφαρμοστούν τα κατάλληλα μέτρα ελέγχου και πρόληψης της νόσου, προστατεύοντας με τον πλέον επιτυχημένο τρόπο τη δημόσια υγεία και την υγεία των ζώων.

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