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The challenge of West Nile virus in Europe: knowledge gaps and research priorities

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West Nile virus (WNV) is continuously spreading across Europe, and other continents, i.e. North and South America and many other regions of the world. Despite the overall sporadic nature of outbreaks with cases of West Nile neuroinvasive disease (WNND) in Europe, the spillover events have increased and the virus has been introduced into new areas. The high genetic diversity of the virus, with remarkable phenotypic variation, and its endemic circulation in several countries, require an intensification of the integrated and multidisciplinary research efforts built under the 7th Framework Programme of the European Union (FP7). It is important to better clarify several aspects of WNV circulation in Europe, including its ecology, genomic diversity, pathogenicity, transmissibility, diagnosis and control options, under different environmental and socio-economic scenarios. Identifying WNV endemic as well as infection-free areas is becoming a need for the development of human vaccines and therapeutics and the application of blood and organs safety regulations. This review, produced as a joint initiative among European experts and based on analysis of 118 scientific papers published between 2004 and 2014, provides the state of knowledge on WNV and highlights the existing knowledge and research gaps that need to be addressed with high priority in Europe and neighbouring countries.

Introduction

West Nile virus (WNV) is an African flavivirus originally maintained in sylvatic cycles mostly between mosquitoes and birds. Since the 1950s, there has been evidence of circulation outside its original ecological niches [1]. Relatively large outbreaks of West Nile neuroinvasive disease (WNND) have been recorded in humans and/or horses in an increasing number of areas. WNV infections have been identified in many European and Mediterranean countries (Figure 1) [1,2]. The increasing incidence of WNND, the appearance of new foci, and the endemic virus circulation in temperate areas have promoted research and innovation efforts financially supported by the European Commission (EC) under the 7th Framework Programme (FP7). However, knowledge gaps remain on several important aspects of virus ecology, biology, and pathogenicity. Together with the lack of safe vaccines and specific therapeutic treatments for humans, this limits our ability to efficiently predict, prevent and control WNV infections, with increasing costs due to the needs of guarantee the safety of blood transfusion and organ donation [3]. There are also rising problems associated with the loss in efficacy and increased resistance to many commercial insecticidal products used to suppress mosquito populations [4]. This requires the intensification of research efforts for emerging vector-borne infections, such as those caused by WNV and other flaviviruses, and the maintenance of the existing research capacity.

Therefore, the coordinators and representative members of four EC FP7 European funded projects on WNV and other mosquito-borne viral diseases (EuroWestNile, EDENext, Wings, Vectorie) met in Madrid on several occasions to review and discuss WNV knowledge advances and identify research gaps to be addressed, under a 'One Health' perspective, with high priority.

Methods

The expert group integrated their expert knowledge and opinion with a scientific literature review. Following a search in the databases ISI Web of Knowledge and PubMed, using defined qualifiers for the viral infection [(WNV)], the disease [(WNND)], host [(humans], [birds], [horses)], geographical location [(Africa], [Europe], [Mediterranean basin)] and issue [(virology], [genomics], [epidemiology], [ecology], [pathology], [diagnostics], [vaccine], [control], [review]). In the search, the period of publication was limited to publications from January 2004 to October 2014. Inclusion criteria were based on title, abstract and year of publication, leading, if relevant to the above mentioned qualifiers, to retrieval and analysis of the full paper. Exclusion criteria were based on the year of publication and on the novelty of the information provided choosing those most up to date on the same subject and those including the previous in the reference list. Only articles with the latest findings on a specific subject were included and those older than the period covered were discarded.

A total of 118 scientific publications were then chosen among the 513 initially retrieved.

Epidemiology of West Nile virus in Europe and neighbouring countries

Circulating West Nile virus strains

The re-emergence of WNV in Europe and neighbouring countries after 1990 led to an intensified surveillance for WNV infection not only in humans, but also in horses, birds, and mosquitoes in several areas. This has resulted in the detection and/or isolation of many different strains of WNV, eventually classified as up to seven (nine) different genetic lineages [5-10] (Table 1, Figure 2 A-C). There is a growing number of WNV lineages and to address inconsistent numbering in the literature we suggest in Table 1 and Figure 2 a harmonised WNV lineage numbering.

The most widespread WNV lineages include lineage 1, clade 1a, belonging to the Mediterranean and former eastern European subtype, and lineage 2, which emerged in central Europe in 2004, and dispersed from Hungary to the eastern part of Austria and to southern European countries [11,12]. Following an independent introduction, another lineage 2 strain was detected in 2004 in Rostov Oblast, southern Russia [13]; subsequently, this virus strain has been responsible for outbreaks of WNND in Volgograd Oblast southern Russia since 2007, and in Romania since 2010 [14,15]. Phylogenetic analyses revealed that all European WNV lineage 1 and 2 strains are derived from a limited number of independent introductions, most likely from Africa, followed by local spread and evolution [5,15-20]. Other lineages identified but not associated so far with human or animal diseases include WNV lineage 3, also known as Rabensburg virus, first isolated in 1997 in South Moravia (Czech Republic) in *Culex pipiens* and *Aedes rossicus* mosquitoes in Czech Republic, and named after the nearby Austrian city of Rabensburg [6], WNV lineage 4a, Krasnodar virus, first detected in a *Dermacentor* tick and then in mosquitoes and frogs in southern Russia [7], putative WNV lineage 4b, identified in 2010 mosquitoes in southern Spain [8], and lineage 4c detected in *Uranotaenia unguiculata* mosquitoes in Austria in 2013 [9].

In recent years, the availability of whole WNV genome sequences of European origin, necessary for diagnostic and molecular epidemiology and for better refining WNV taxonomic classification, has improved. A WNV strain bank and associated large genomic database has been built by the EuroWestNile consortium to facilitate this availability. The biobank and database are now available for the scientific community (www.eurowestnile.org).

Analyses of full genome sequence data have shown that the virus, notwithstanding its high genetic stability, can adapt to new ecological niches through mutation and selection events [21]. In addition to this plasticity, the presence of other flaviviruses infecting mosquitoes, birds and humans, may have important consequences not only for WNV ecology, epidemiology and pathogenicity, but also for diagnostics, surveillance and control strategies [22].

West Nile lineages and human cases

Most humans infected with WNV remain asymptomatic and only approximately 20–40% develop symptoms. The vast majority of clinical manifestations are a mild influenza-like illness, defined as West Nile fever (WNF); severe neuroinvasive disease (WNND) occurs only in <1% of the infected patients. WNND usually encompasses three different syndromes: meningitis, encephalitis, and acute flaccid paralysis [2 and references herein]. According to the European Centre for Disease Prevention and Control (ECDC), the total number of WNND cases reported from 2010 to 2013 was particularly high in Greece, with 262 cases of WNND notified in 2010 (incidence 2.34/100,000), 100 in 2011 (incidence 0.90/100,000), 161 in 2012 (incidence 1.45/100,000), and 86 in 2013 (incidence 0.78/100,000) (unpublished data).

WNV lineage 2 strains belonging to the Hungarian clade were responsible for the outbreaks in Greece [23,24] and in other central European countries, including Serbia [25-27], where 71 (incidence 0.98/100,000) and 302 (incidence 4.91/100,000) human infections were notified in 2012 and 2013, respectively. Autochthonous

human cases of infection with this WNV strain were identified in recent years in Hungary, Austria [28], Croatia [29,30], Albania, the former Yugoslav Republic of Macedonia, Kosovo* and Montenegro. Closely related WNV lineage 2 strains of the same Hungarian clade have also been detected in Italy since 2011 [31-33], leading to a large outbreak with 69 notified human infections (incidence 0.12/100,000) in 2013 [34], and in the Czech Republic in 2013 [35]. The epidemiological situation in Italy is, however, more complex than in other countries [36]. WNV lineage 1 strains of the western Mediterranean subtype, including the Livenza strain that caused an outbreak in the area in northern Italy in 2012 [19,37] that was later affected by the WNV lineage 2 strain in 2013, have been autochthonous in Italy and co-circulate with WNV lineage 2. WNV lineage 1 strains classified as western Mediterranean subtype circulated also in southern Spain [20], where sporadic human infections were reported in 2010.

In Turkey, human cases of WNV infection were reported in 2010 (47 cases, incidence 0.06/ 100,000) and in 2011 (five cases, incidence 0.01/100,000) [38]. WNV strains obtained between 2011 and 2013 from humans, horses and mosquitoes across Turkey proved to be closely related to 'old' lineage 1 strains from sub-Saharan Africa [39]. Israel, a highly WNV-affected Mediterranean country [40], noted over 100 cases diagnosed in 2010, 33 in 2011, 83 in 2012, and 63 in 2013 (incidence rates per 100,000 not available) [40]. Finally, the southern Russian/Romanian WNV lineage 2 strain [15] has led to large outbreaks of WNND in humans in southern Russia in each season from 2010 to 2013 (419 cases in 2010, 137 in 2011, 447 in 2012, and 177 in 2013, incidences rates per 100,000 not available), and in 2010 Romania, when 57 human infections (incidence 0.28/100,000) were identified [14].

Ecology

Mosquito vectors

Several mosquito genera are competent for WNV transmission; however, mosquitoes belonging to the *Culex pipiens* complex and their hybrids play a central role in modulating the virus circulation and the seasonal shifts among birds and humans [41]. In addition to *Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus* have been identified as important WNV vectors [42], while the role of other species, including the *Aedes albopictus*, needs to be better evaluated.

Introduction, transmission and spread

Introduction of WNV into new areas is generally considered to be initiated by migratory birds while residential and synanthropic birds may contribute to virus dispersal into larger areas, as well as to the following establishment and spread in certain areas [43,44]. However, the relative importance of dispersal of WNV through infected mosquitoes rather than by infected birds is unknown. The introduction event may or may not be followed by an amplification phase depending on several coincidental factors, ranging from the birds' and vectors' competence, abundance and community assembly, to the local environmental condition including climate and landscape features [45-47]. One recognised important factor for WNV amplification and the following risk of transmission to humans is the feeding behaviour and host selection by the mosquito vectors. Based on preliminary research carried out in Italy and Spain, only few bird species seem to play a major role as blood donor for the mosquitoes [42,48,49]. Unfortunately, the reservoir competence for many European bird species is still unknown. Furthermore, mass mortality of highly susceptible species (such as corvids or other species) is less frequently observed in the Old than in the New World although some species, as the jackdaws (*Corvus monedula*) or other could potentially function as sentinel [50].

Virus persistence, silent circulation and spillover

Mechanisms of virus persistence in animal hosts in Europe are still unknown, but they could possibly lead to the development of persistent, chronic infections in certain individuals as seen in studies carried out in the United States [43,51]. In fact, in the New World, WNV, once established, circulates in enzootic cycles among few most competent mosquito species and their preferred feeding hosts [43,48,51]. The circulation of WNV in Europe may occur silently for several months, or even years, before the spillover event occurs [52]. One or more bird species may be involved in the amplification chain, some being important in the maintenance of the sylvatic cycles, while others (that might not be the same) may be involved in the periurban and urban cycles. Following spillover and following outbreak a subsidence phase may occur as a consequence of the rise of herd immunity in the reservoir birds, that is dependent on host longevity and the rate of recruitment of young individuals into the host population or the depopulation of highly susceptible bird hosts [43,51]. Unfortunately, the knowledge on herd immunity to WNV for many bird species of the Old World is still lacking.

WNV diversity and interactions with other flaviviruses

A wide spectrum of WNV lineages and strains with different potential pathogenicity and virulence thrive among other related flaviviruses with overlapping ecology such as Bagaza virus (BAGV) and Usutu virus (USUV)[52,53]. In this scenario, a previous infection of the WNV amplifying bird hosts with a low pathogenic strain of WNV or with a closely related flavivirus may confer some degree of cross-immunity which might reduce the amplification of any new WNV strain introduced into the same area [22]. This may explain, for instance, the scarcity of human WNV cases detected in Spain, where rates of WNV infection in birds are high and that may be due to the co-circulation of WNV lineage 6 [8] or other flaviviruses, that induce the production of potentially cross-protective antibodies in both

humans and birds, as reported for example for USUV [54].

However, co-infection may occur also in the mosquito vectors and therefore WNV replication could be in some way affected by previous infection with other co-circulating flaviviruses of the same antigenic group or even more distant viruses so that the epidemiological picture may change from one site area to another [55]. Although some investigations on viral interference have been started with one project (EuroWestNile), we are still at the beginning of our understanding of the consequences of viral co-infections both in the host and in the vectors, and therefore research in this field is now of high interest.

Consequences for WNV surveillance in Europe

Passive surveillance programmes focusing only on the analysis of dead birds may fail to detect ongoing WNV circulation in Europe. It is advisable to combine conventional surveillance activities with actively monitoring seroconversion in sentinel and/or wild resident birds and horses [56-59].

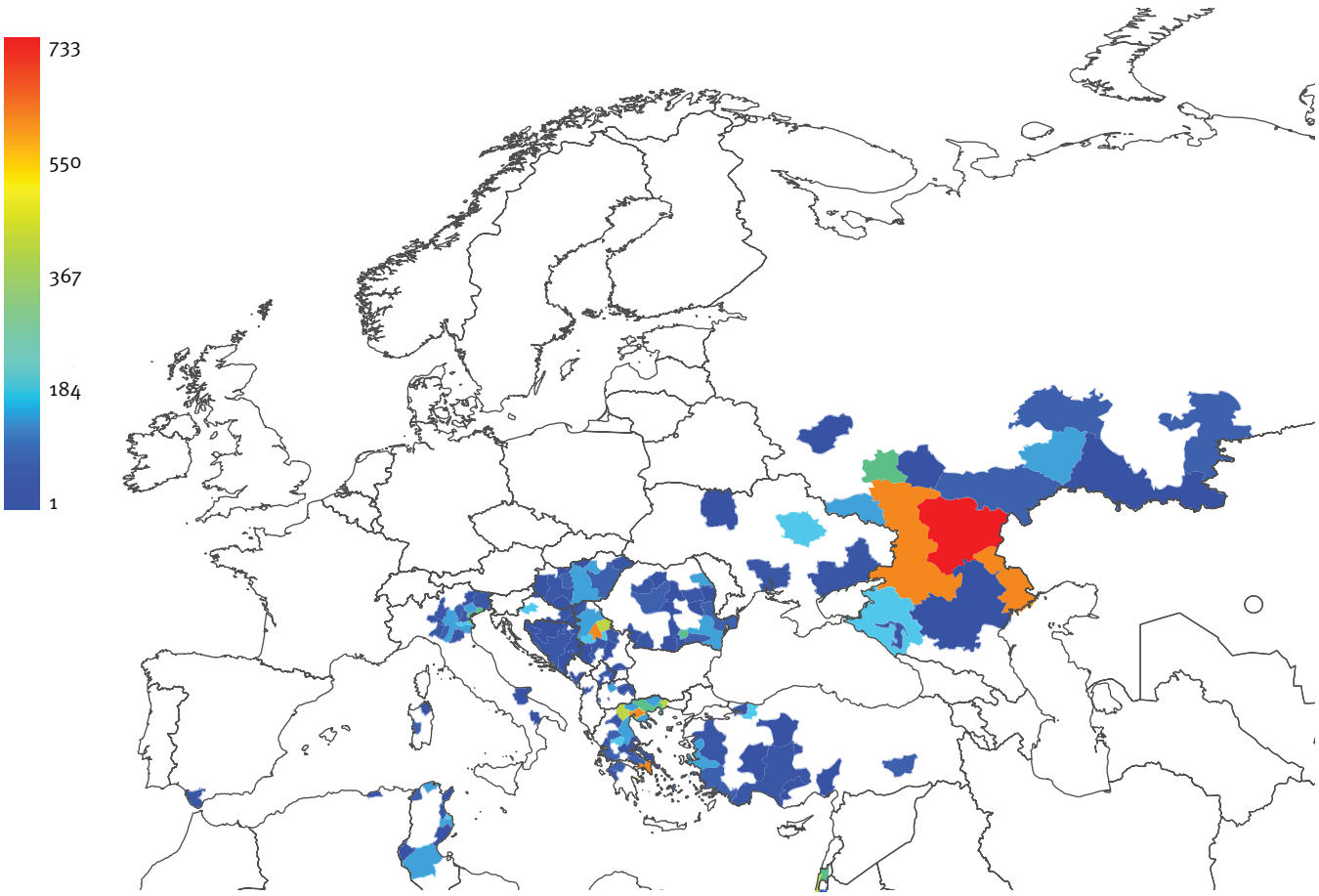
Pathogenicity and other phenotypic traits

Studies using animal models, mainly mice, have provided insights into WNV pathogenesis. Intra-peritoneal injection of WNV into mice generally leads to encephalitis and other neurological signs resembling those observed in humans with WNND [60]. This model provides researchers with a simple method enabling phenotypic characterisation of WNV strains for neuropathogenicity and neuroinvasiveness. Through the integration of this information with analysis of genetic changes occurring in field WNV isolates and reverse genetics using infectious clones, molecular chimeras or other strategies, it is possible to identify determinants of virulence [61-63]. However, the mouse model has important limitations and thus alternative animal models are highly desirable. An attempt of this kind, using one day old chickens, has recently been reported [64]. In addition, bird models of WNV infection provide information about the course of the infection in natural hosts [65 and references herein] and, consequently, about key aspects of WNV epidemiology.

Genetic changes in different geographic WNV variants might allow to identify the link with phenotypic traits related to virulence, amplification, transmissibility to

Figure 1

Cumulative number of human cases of West Nile virus infection in Europe and neighbouring countries, 2010 to 2013



Data source: European Centre for Disease Prevention and Control (ECDC).

Table 1

Overview of West Nile virus lineages and suggested lineage numbering

Suggested lineage numbering	Other lineage labelling in the literature	Representative strain	GenBank accession number	Note	Reference
Lineage 1a	Lineage 1	NY99-flamingo382-99, New York, 1999	AF196835	Most widespread WNV lineage	Lanciotti et al., 1999 [115]
Lineage 1b	Lineage 1	Kunjin MRM61C, Australia, 1960	D00246	Kunjin virus strains, Australia	Coia et al., 1988 [116]
Lineage 1c	Lineage 5	804994, India 1980	DQ256376	Only found in India	Bondre et al., 2007 [117]
Lineage 2	No	B956, Uganda 1937 (oldest WNV strain; WNV prototype strain)	AY532665	Second most widespread WNV lineage	Smithburn et. al, 1940 [118]
Lineage 3	No	Rabensburg virus 97-103, Czech Republic 1997	AY765264	Only found in central Europe	Bakonyi et al. [6]
Lineage 4a	Lineage 4	LEIV-Krnd88-190, Russia 1998	AY277251	Originally isolated from Dermacentor ticks	Lvov et al. [7]
Lineage 4b	Lineage 6 / Lineage 7	HU2925/06, Spain	GU047875	Only partial sequence available	Vázquez et al. [8]
Lineage 4c	Lineage 9	WNV-Uu-LN-AT-2013, Austria 2013	KJ831223	Identified in Uranotaenia mosquitoes	Pachler et al. [9]
Lineage 5	Lineage 6	Kunjin virus KUN MP502-66, Malaysia 1966	GU047874, HQ840762	Only partial sequences available	Vázquez et al. [8]
Lineage 6	Lineage 7	Dak Ar D 5443, Senegal	EU082200	Koutango virus	NA
Lineage 7	Lineage 8	ArD94343, Senegal 1992	KJ131502	Only partial sequence available	Fall et al. [10]

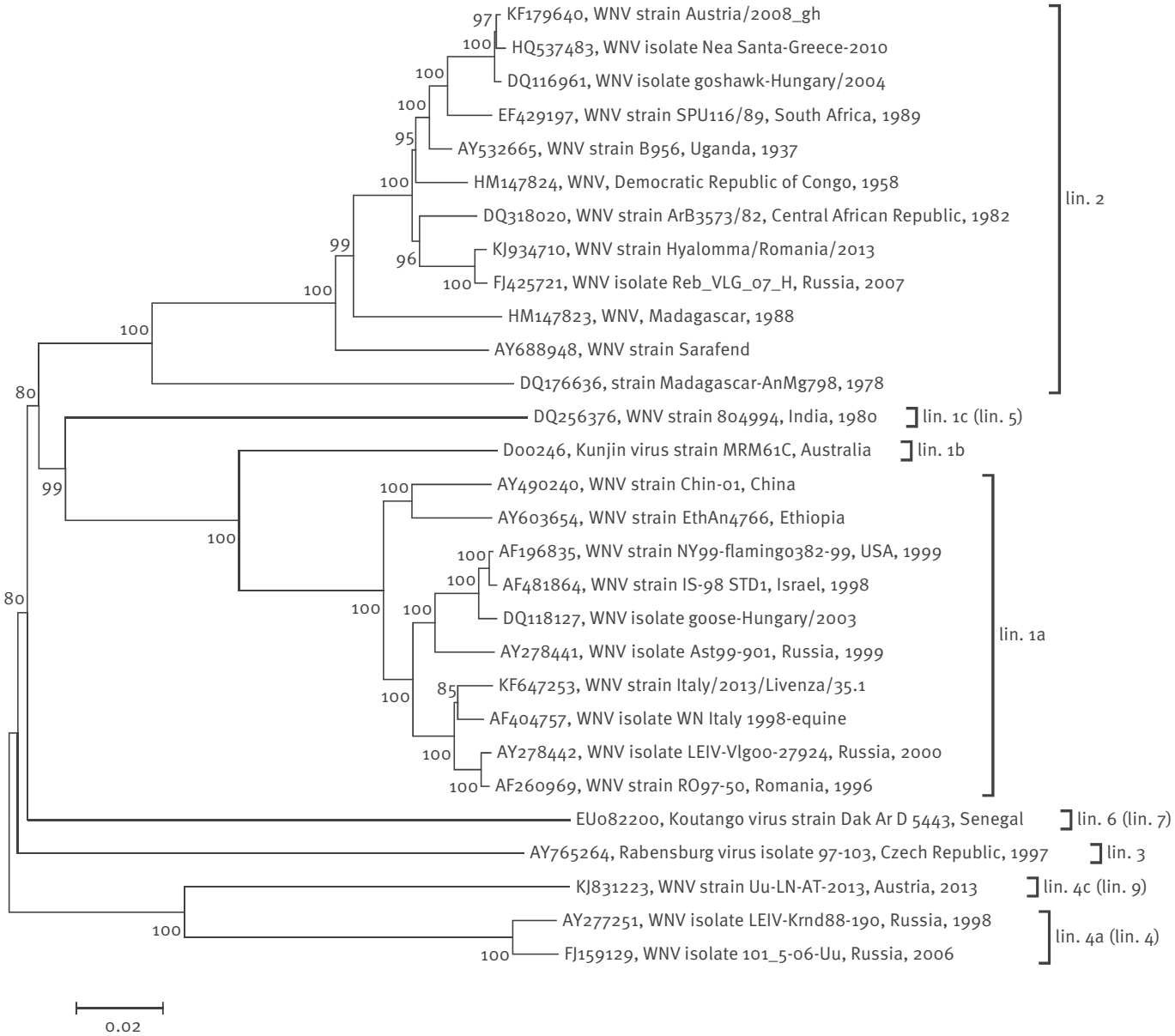
NA: not available; WNV: West Nile virus.

mosquitoes, and/or persistence, expressed in infected birds. Studies in avian models of WNV infection began in the US shortly after the first occurrence of WNV cases in 1999, and identified passerines as the group of birds developing higher and longer viraemias [66]. In contrast, studies testing the effect of European WNV strains in bird species indigenous to Europe have only been started recently [67-69].

Table 2 lists widely recognised genetic changes associated with virulence/attenuation, confirmed by viral cDNA clone mutagenesis/chimeras and using animal models of WNV infection [70-78]. Point mutations leading to amino acid changes either in structural (E) or non-structural (NS2A, NS3, NS4B) proteins result in attenuated phenotypes in different models. Pathogenicity appears also to be influenced by changes at both 5' and 3' non-coding regions. Interestingly, the WNV genome is relatively flexible as it can tolerate a number of changes in its sequence that do not seem to affect pathogenicity. However, generalisation of these findings could be misleading. The spectrum of WNV strains to which they can be applicable needs to be defined, as the phenotypic effect of each of the changes described for one given strain might not affect all WNV strains equally. Care should be exercised not to extend blindly the results observed in a given model to other susceptible species. For instance, the T249P mutation at the NS3 increases pathogenicity of a Kenyan WNV L1a strain for American crows (*Corvus brachyrhynchos*), and the opposite mutation reverts the high pathogenicity observed for the NY99 WNV strain in this species. However, this effect applies for American crows and not for house sparrows [79]. Moreover, Mediterranean WNV L1a strains with the $NS3_{249}P$ genotype did not show higher pathogenicity than their $NS3_{249}T$ counterparts either in mice [80], in the red-legged partridge (a bird species indigenous to southern Europe) [67], and in the house sparrow [68], although it has been recently shown that this mutation can modulate WNV pathogenicity of certain Mediterranean WNV strains for the European corvid, *Corvus corone* (carrion crow) [81]. Interestingly, the Greek WNV lineage 2 strains from the outbreaks in 2010 and 2011, involving human morbidity and mortality, have been shown to bear the $NS3_{249}P$, contrary to their likely precursors from central Europe and the WNV strains that caused outbreaks in Serbia and in Italy, which have a histidine residue instead of proline at this site [58]. Despite suggestive, the association between the high pathogenicity observed in human outbreaks and the presence of $NS3_{249}P$ in these Greek lineage 2 WNV strains need to be assessed experimentally. Also, phenotypic assessment of WNV strains should not be limited to pathogenicity but should take in consideration other phenotypic traits, such as host competence (capacity of a given host species to transmit the virus efficiently to a mosquito feeding on its blood)/transmissibility. Variations in host competence have been reported for different WNV strains in the house sparrow [68,69], which might help to explain, at least in part, the different epidemiological patterns observed in the New versus the Old World.

Figure 2A

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]

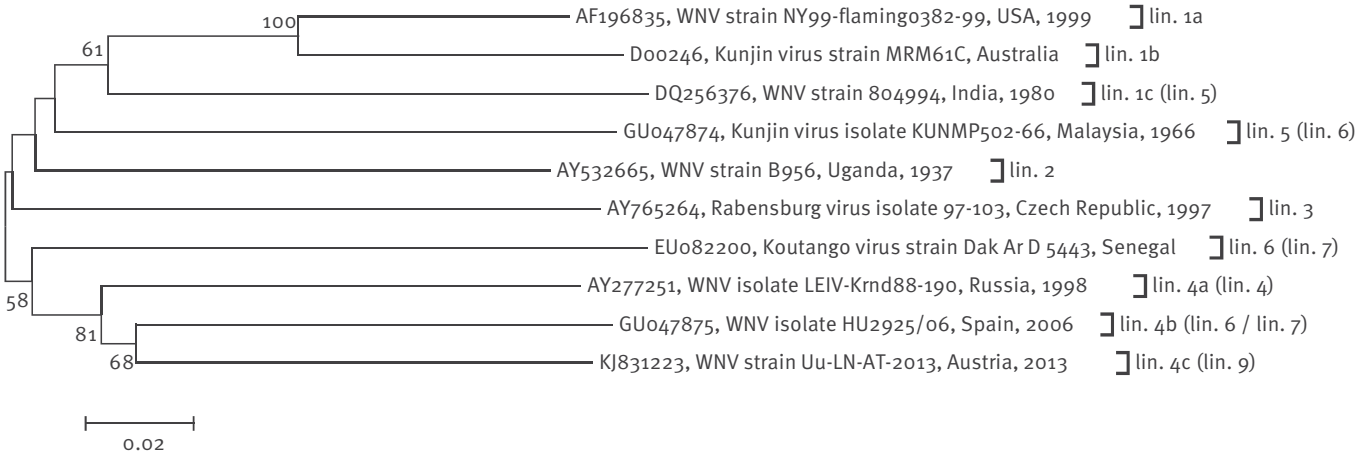


ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

[a] Other lineage labelling in the literature is given between brackets.

Figure 2B

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]

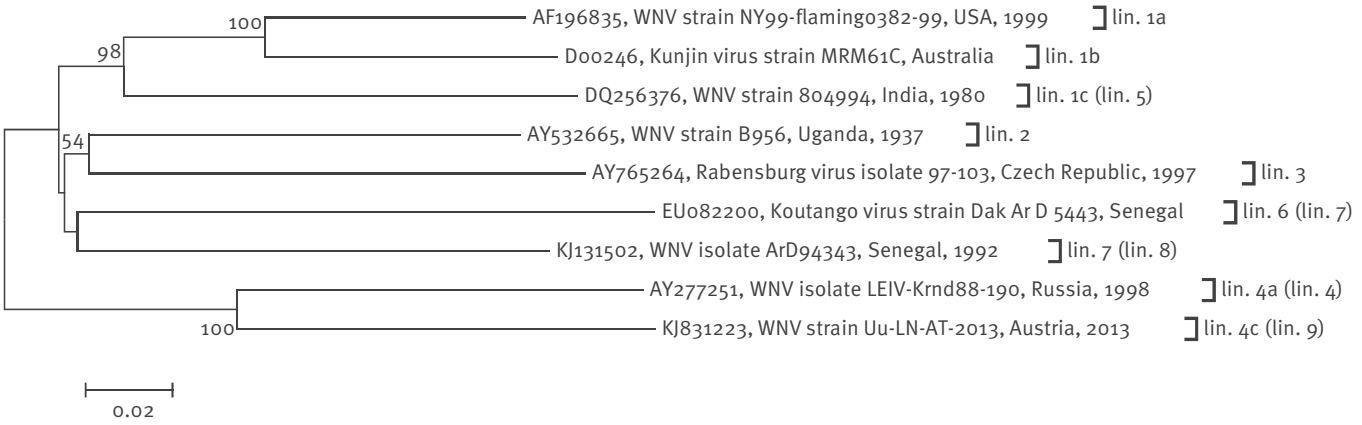


ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

[a] Other lineage labelling in the literature is given between brackets.

Figure 2C

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]



ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

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Diagnosis

The laboratory diagnosis of acute WNV infection is based on both the detection of WNV RNA in blood and cerebrospinal fluid (CSF) or virus isolation in cell culture from serum samples (direct diagnosis) and on the demonstration of a specific immune response against the virus (indirect diagnosis), recently reviewed by [82] and [83].

Detection of viral RNA

Detection of WNV RNA in biological specimens represents a rapid method to unambiguously prove the infection with WNV [84]. Different PCR-based protocols have been developed to amplify minimal amounts of WNV RNA [85]. However, viraemia is short-lived in dead-end hosts and already declining substantially once the symptoms begin. Hence, detection of viral genomes becomes increasingly challenging over time. As a consequence, WNV RNA is generally not detectable in the blood of patients with symptomatic infection [86]. Additionally highly sensitive nucleic acid amplification-based methods may rarely provide false-positive results by cross-reaction with other flaviviruses [87]. Figure 3 delineates a timeline of clinical and diagnostic markers during human WNV infection.

Recent data demonstrated that WNV RNA can be detected in urine much longer and at higher concentrations than in blood or CSF in individuals with WNF or WNND [86,88]. The duration of WNV RNA detection in urine seems dependent on the course of disease and could last for 20 days or longer [86].

Similar results were observed in experimentally infected monkeys and hamsters [89,90]. The virus excreted in urine is infectious, since it can be isolated in cell culture from urine specimens collected from patients with acute infection [91].

Implementation of WNV RNA testing and isolation from urine samples in routine protocols for WNV diagnosis demonstrated the utility of these tests for the confirmation of cases [30,86,88,91]. Although PCR methods have been developed to detect both major WNV lineages [92], a first external quality assessment (EQA) study in 2006, revealed that many laboratories had problems in detecting genomes of WNV lineage 2 [93], an issue of high concern since lineage 1 and 2 viruses are co-circulating in Europe. In a recent second EQA in 2011, the participating laboratories had improved significantly regarding this aspect [87]. PCR multiplexing can be useful in this respect, and recently a multiplex PCR method able to detect and differentiate WNV lineages 1 and 2 and USUV has been developed [94]. It is also essential that current PCR methods are constantly being checked and updated for their sensitivity and suitability to detect newly emerging WNV strains to cope with the observed genetic variability of European WNV strains.

Antibody detection

Antibodies against WNV start to appear about four to seven days following infection and IgM antibodies are already detectable at early time points after symptoms onset [86] (Figure 3). Thus, the diagnosis of WNV infection generally relies on the demonstration of specific antibodies against WNV in serum or CSF.

The major problem with most serological diagnostic tests for WNV is cross-reactivity with infections caused by other flaviviruses, and the last EQA on WNV serology organised by the European Network for Diagnostics of Imported Viral Diseases (ENIVD) also showed the limits and needs for improvement [95]. Therefore, considerable effort is being made to develop antigens and/or test formats which can be used for specific detection of anti-WNV antibodies [reviewed in 83]. These also have to take into consideration the heterogeneity of human antibody profiles to WNV infection [96]. Furthermore, several flaviviruses have been found in areas affected by WNV, including BAGV, tick-borne encephalitis virus TBEV , and USUV [22,53,97,98].

Taking into account these problems, the European Union case definition for WNV infections demands confirmation in cases with IgM detection in serum by virus neutralisation test (VNT) [99], which today is the gold standard diagnostic method for flavivirus serology (reviewed in [83]). However, VNTs are time-consuming and require a biosafety level (BSL)-3 laboratory. Monoclonal antibody panels with strong and specific reactivity to TBEV, USUV and WNV were recently developed [100], which will likely enable the development of improved immunoassays for the detection and differentiation of flavivirus infections. Furthermore, monoclonal antibodies could be used for the development of competitive tests for the detection of different types of anti-WNV antibodies suitable for the range of WNV host species, including many species of wild birds and susceptible mammals [101]. Alternatively, the use of mutant forms of viral proteins leads to a minimisation of antibody cross-reactivity and enables the serological differentiation of flavivirus infections [102].

Vaccines

The persistent long-term effects seen after resolution of acute WNV infection in humans, the emerging threat that WNV poses to Europe and the placement of WNV on the list of bioterrorism agents, makes the development of a safe and effective vaccine for humans an urgent priority [103]. In fact, while several vaccines are already available for horses [104-106] and other currently under development due to the impact of WNV infection on the horse industry, no vaccine is available for humans.

Towards the production of a human vaccine

The increasing understanding of WNV pathogenesis and correlates of protection in animal models pave the way to a more rational design of candidate vaccines. Studies performed in mice elucidated some of

Table 2

Non-exhaustive list of genetic determinants of West Nile virus pathogenicity identified in animal models

Genome region	Genetic change	Phenotypic effect(s)/mechanism(s) involved	Phylogenetic group	Animal model used	Experimental system	References
E	Various, on the 154-NYS(T)-156 (N-glycosylation) motif	N-glycosylation site abolished; unstable E-peptide fusion; decreased viral replication.	Lineages 1 (clades a and b) and 2	Mice	cDNA clone mutagenesis	[70,71]
NS2A	A30P	NS1' (NS1 extension) abolished; disruption of NS1-mediated immune evasion mechanisms.	Kunjin (Lineage 1, clade b)	Mice	cDNA clone mutagenesis	[72,73]
NS3	T249P	Increased virogenesis, efficient replication at higher temperatures.	Lineage 1, clade a	American crow	cDNA clone mutagenesis	[74]
NS4B	C102S P38G E249G	Abrogation of evasion from host innate immunity (IFN α/β response); decreased helicase activity (decreased virus replication).	Lineage 1 clade a	Mice	cDNA clone mutagenesis	[75,76]
3'UTR	Unknown	sfRNA abrogation.	Lineage 1, clade b	Mice	cDNA clone mutagenesis	[77]
5'UTR	5-AAT/TTG-52	Unknown	Lineage 1, clades a and b	Mice	cDNA clone mutagenesis and chimeras	[78]

WNV: West Nile virus.

The list represents widely recognised genetic changes associated with WNV virulence/attenuation that have been shown to occur in natural WNV isolates.

the mechanisms of protection, such as the role of the adaptive immune responses in mitigating and preventing development of disease. This is exemplified by the observation that transfer of WNV-specific antibodies to naïve mice protected the animals against development of severe neurological disease [107]. Several lines of evidence indicate that the B cell response after infection with WNV is predominantly directed to non-neutralising epitopes located in domain I and II of glycoprotein E. The most potent neutralising epitopes are located in domain III (the receptor-binding ligand). For the development of a WNV vaccine for humans it is important to understand how vaccination could increase the longevity of B cells that produce these potent neutralising antibodies. As the probability of neuroinvasion in the animal host appeared to correlate with the level and duration of viraemia, it is conceivable that persistence of neutralising antibodies is sufficient to protect against severe disease [108]. Although T cells play a role in eliminating an established infection, thereby reducing the antigen load, it is believed that antibodies are more important for vaccine-induced protection. It is worth noting that both CD4+ and CD8+ T cells have been shown to contribute to control of WNV infection in animal models but it cannot be excluded that T cells may also cause disease [109].

Several platforms have been used to develop candidate vaccines against WNV for humans [109]. Various vaccine candidates have been shown to be highly effective in preventing fatal disease in mice and horses [110-112]. Long-term immunogenicity and safety, however, are some of the most important problems in current veterinary vaccines against WNV and potential candidates for use in humans. The regulatory authorities as well as public opinion demand safe and well characterised vaccines. Therefore, an effective WNV vaccine should stimulate protective and long-term immunity, preferably after a single dose, and should be free of significant side effects, especially in the elderly and immunocompromised individuals. Research on the use of several platforms for development of a safe and effective vaccine against WNV lineages 1 and 2 was an important topic in several European consortia and yielded promising candidate approaches.

Knowledge gaps and research priorities

Although significant progress in the scientific knowledge on WNV in Europe has been made through projects funded by the FP7, several knowledge gaps still limit our ability to properly forecast changes in the risks of outbreaks to occur or to prevent and control virus transmission to humans and animals.

Knowledge gaps on the introduction and spread of WNV in Europe

Our knowledge on how, when and from where WNV is introduced into Europe is still very limited today. The role of migrating birds on WNV translocations is recognised, however, many important questions still remain unanswered e.g. the geographic origin of each introduction of WNV into Europe, the relative importance of the introduction of infected vectors rather than infected birds, and the detailed transmission mechanisms involved in virus establishment into new areas. For these reasons, better knowledge on the WNV ecology, vectors and virus strains circulating currently in Africa will be of utmost importance. Key factors leading to virus amplification and spread to neighbouring areas are also essentially unknown. Furthermore, we know that the virus may disappear from a previously infected area but we ignore the key factors leading to

its extinction. Spillover events are increasing in numbers and expand geographically over time in Europe, however, our knowledge on the factors triggering such events continues to be rather poor. We do not know in most cases which are the key amplifying bird host and the bridge vector species involved. Moreover, we do not know the consequences of interventions such as massive culling of species considered as 'pests' and the contribution of factors such as species community composition under variable climatic and environmental conditions at local level. With this level of eco-epidemiological uncertainty, building-up accurate predictive models of WNV outbreak risk is a complex undertaking, although progress has been made in this direction [46,47,113]. Epidemiological models rely on good knowledge on basic epidemiological information of the virus and the disease it causes, which is still lacking in many cases, as outlined above. Filling this gap will not be an easy task; for instance, competence for WNV transmission of each host and vector species must be calculated experimentally for each viral strain, but given the heterogeneity of WNV found in Europe, this work will require a huge effort even if restricted to few representative strains and host/vector species.

Knowledge gaps on the impact of the presence of other flaviviruses on WNV epidemiology

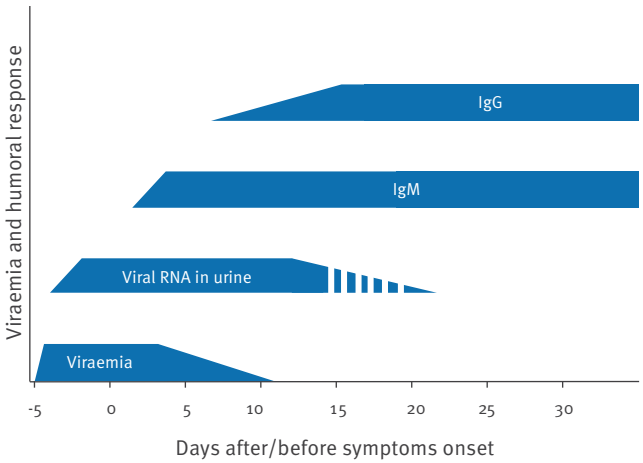
Another important knowledge gap is the impact that other co-circulating flaviviruses (and perhaps arboviruses) may have on WNV epidemiology in Europe. Cross-immunity between closely related flaviviruses might lead to some degree of cross-protection in the animal hosts, or, even enhance infection in certain cases. Further assessment of the consequences of viral interference on the immune response both in hosts and vectors in Europe is needed. Realistic WNV models should also incorporate these effects. The diversity of flaviviruses circulating in Europe, including mosquito-only flaviviruses as well as newly emerging flavivirus strains, represent another challenge for WNV research. Europe's preparedness to face emerging flavivirus threats depends largely on the availability of large datasets integrating genomic characterisation and analysis of all the strains isolated in Europe and neighbouring areas, including Africa. These datasets, together with the already started bank of viral strains, will be of paramount importance for the development of efficient molecular tools enabling proper flavivirus detection, identification and classification, along with studies on the origins of the different flaviviruses emerging in Europe and their evolutionary history.

Knowledge gaps on mosquito control

There is a need to strengthen the entomological capacity in Europe to improve surveillance, and risk assessment, and to identify the most appropriate and environmental friendly control strategies especially within hot spots of arbovirus emergence and spread in the Old World. The number of products available for mosquito control has been reduced in recent years. New insecticides and repellents are urgently needed since those available are losing efficacy. There is a clear need to define and evaluate the effectiveness of integrated mosquito control strategies and involve the public in mosquito source reduction. Models to support decision in mosquito control and management are also urgently required.

Figure 3

Viraemia and humoral immune responses against West Nile virus



Knowledge gaps on the identification of virulence factors

Virus strain characterisation, in particular, virulence studies and identification of virulence determinants demand proper experimental animal models. Rodents (mice, hamsters) used as surrogate incidental mammal host model still need some standardisation and the same applies to wild birds, used as susceptible, reservoir and amplification host models useful to obtain data on virus pathogenicity and transmission. Suitable animal models would not only constitute an invaluable tool for virus characterisation, but also enable studies on co-infection, cross-protection, vaccine efficacy, therapeutic testing, the effect of stress and immunosuppression during the course of infection, among others.

Knowledge gaps on laboratory detection

The challenge in diagnostics is the WNV diversity in Europe and the need to continuously check the effectiveness of existing laboratory tests for the detection of newly arising strains or antibodies to them. This requires a considerable effort from all WNV diagnostic laboratories involved. The main gap in WNV serology is to develop new generation immunoassays avoiding cross-reactions with different flaviviruses and enabling good flavivirus differentiation, minimising the need for confirmation by the gold standard VNT. For that, large panels of well-characterised monoclonal antibodies to different flaviviral epitopes would be invaluable. Development of markers of early/mature immune response is also desirable. In the veterinary field, serological tests differentiating infected from vaccinated

individuals are largely awaited. Where possible, inclusion of different types of antigens in the immunoassays would help to avoid false negative results due to heterogeneity of the humoral immune responses to WNV. In molecular diagnostics, new methods enabling rapid differential diagnosis of flavivirus infections, preferably in multiplex formats, are needed. New samples for virus detection and isolation (for instance, urine in humans, or feathers in wild bird surveillance) need further validation to assess their impact on diagnostic sensitivity and specificity, also, when possible, with different WNV strains.

Knowledge gaps on therapy and prevention

The lack of specific treatment and of human vaccines constitutes another gap in the mitigation of WNND. Together with the high susceptibility of the elderly, this renders the disease of great societal impact because of the ageing of the European population. The spread of WNV, even in the absence of clinical cases of WNND, imposes a significant economic burden for European public health due to the need to guarantee blood and organ transplantation safety.

Gaps in vaccine development include safety issues, especially in target susceptible populations such as the elderly and immunocompromised individuals. Due to the unpredictable regional spread of WNV outbreaks, clinical effectiveness testing of WNV vaccines in humans seems difficult. In combination with the yet limited market size, this has so far prevented further development of vaccine candidates by the pharmaceutical industry. Therefore, in order to move one or more of the existing (and promising) candidate vaccines towards clinical development, there needs to be a joint evaluation between industry, researchers and regulatory authorities. The possible interactions with other available flavivirus vaccines in Europe such as the ones against TBE and yellow fever virus (the latter only used for travellers to endemic areas outside Europe), and interactions with infections by other related flaviviruses, including cross-protection and/or antibody-dependent enhancement of infection, need further clarification.

Conclusion

The unpredictability of the West Nile virus risk for Europe and neighbouring countries is strongly linked to the knowledge gaps on many aspects of its complex ecology, genomic diversity, pathogenicity, transmissibility, diagnosis and control options, under different environmental and socio-economic scenarios. Therefore, there is an urgent need to intensify and continue the research efforts on WNV and other emerging vector-borne infections built under the FP7. In parallel, there is a growing need for a unified, harmonised, real-time epidemiological surveillance of WNV in vectors, humans and animals, especially within hot spots of virus circulation in Europe under the guidance of ECDC and other international organisations.

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Conflict of interest

None declared.

Authors' contributions

AT conceived and coordinated the various contribution to the manuscript; AR and MAJC coordinated, wrote, reviewed and edited the final version of the manuscript; AR, AT, LB, NN, and MAJC drafted the contribution on virology and epidemiology; AR and JF drafted the contribution on ecology; NP, MAJC and PK drafted the contribution on pathogenesis; LB, SU, PC and AM drafted the contribution on diagnosis; BM and NS drafted the contribution on vaccine; AR and MAJC drafted the contribution on knowledge gaps. All contributors participated in the final revision of the manuscript.

*Note

This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.

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