Antibody responses in humans infected with newly emerging strains of West Nile Virus in Europe

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Antibody Responses in Humans Infected with Newly Emerging Strains of West Nile Virus in Europe

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Abstract

Infection with West Nile Virus (WNV) affects an increasing number of countries worldwide. Although most human infections result in no or mild flu-like symptoms, the elderly and those with a weakened immune system are at higher risk for developing severe neurological disease. Since its introduction into North America in 1999, WNV has spread across the continental United States and caused annual outbreaks with a total of 36,000 documented clinical cases and ~1,500 deaths. In recent years, outbreaks of neuroinvasive disease also have been reported in Europe. The WNV strains isolated during these outbreaks differ from those in North America, as sequencing has revealed that distinct phylogenetic lineages of WNV concurrently circulate in Europe, which has potential implications for the development of vaccines, therapeutics, and diagnostic tests. Here, we studied the human antibody response to European WNV strains responsible for outbreaks in Italy and Greece in 2010, caused by lineage 1 and 2 strains, respectively. The WNV structural proteins were expressed as a series of overlapping fragments fused to a carrier-protein, and binding of IgG in sera from infected persons was analyzed. The results demonstrate that, although the humoral immune response to WNV in humans is heterogeneous, several dominant peptides are recognized.

Introduction

West Nile Virus (WNV) is a mosquito-borne flavivirus that was first described in Africa in 1937 and is now endemic to large parts of the tropical and subtropical world. In its natural cycle, WNV cycles between mosquitoes and birds but also can be transmitted to animal species. Clinical manifestations of WNV infections in humans range from no symptoms, to a febrile syndrome, to neuroinvasive disease including meningoencephalitis, and acute flaccid paralysis. The most severe neuroinvasive forms affect ~1% of the WNV-infected humans, primarily the elderly or immunocompromised [1]. The virus reached public attention when it was introduced into North America in 1999, and since has spread over the entire continent [2]. Since the mid-1990s, WNV has emerged in both Eastern and Western Europe, where before it only sporadically caused mild or limited local outbreaks. In comparison, over the last few years, outbreaks with severe disease have been reported in Romania, Hungary, Russia, Italy, Former Yugoslav Republic of Macedonia, and Greece, where the virus is now considered endemic [3–6]. As an example, almost 200 severe WNV infections with 33 deaths were reported in Greece in 2010 [7]. The rate of severe neurologic disease was similar to the one observed in the USA [AP, unpublished]. The positive-stranded RNA genome of WNV encodes for the three structural proteins capsid (C), pre-membrane/membrane (prM/M) and the envelope (E) protein, as well as seven non-structural proteins [8]. The C protein lies within the inner core of the virus and binds to viral RNA to form the nucleocapsid. E is the major surface glycoprotein on the mature virion and functions in several critical events during the viral life cycle, including receptor binding, entry, and endosomal fusion. The small glycoprotein M (and its precursor prM) acts as a chaperone for E protein folding and also is displayed on the virion surface, albeit in a location proximal to the viral membrane [9]. Most WNV isolates are classified into two major lineages, termed lineage 1 and 2, which share ~75% identity at the nucleotide and ~94% at the amino acid level [10]. Unlike the epidemiology in the Americas, where a lineage 1 WNV strain is exclusively detected, there are several strains belonging to different lineages that circulate in Europe. Co-circulation in the same area of different WNV strains belonging to both lineage 1 (clade 1a) and lineage 2 has been reported in Italy [3,11–13], and different WNV lineage 2 strains were responsible
for outbreaks in Greece, Romania, and Russia [4,14]. Co-circulation of WNV of different lineages must be considered for the development of vaccines, therapeutics, and diagnostics.

The humoral immune response to a WNV infection in small animal models is characterized by the appearance of IgM antibodies after 4 to 7 days, with antigen-specific IgG detectable shortly after [15]. Both neutralizing IgM and IgG are important means for controlling the infection and likely limit the viremia that results in WNV dissemination into the central nervous system [16,17]. Antibodies against WNV are also detected by immunofluorescence to diagnose infections, however, cross-reactivity with other circulating flaviviruses can confound diagnosis; thus, functional assays, such as a neutralization test, or nucleic acid based detection systems are required to confirm a diagnosis of WNV infection [18–20].

The human immune response to American WNV infections is being studied extensively. Epitopes for CD8+ T cells and for several human monoclonal antibodies have been mapped [21–24]. B cell epitopes also have been identified by screening selected WNV proteins with sera from birds, rodents, and horses, but only to a limited extent with sera from infected humans [25–28]. In addition, only few data exist as to the immune responses to newly emerging WNV strains.

Herein, we describe a platform to investigate systematically antibody responses to WNV infections using a series of overlapping protein fragments spanning all structural proteins of the virus. This technology was used to study the IgG response of humans to linear B-cell epitopes during WNV outbreaks in Italy and Greece in 2010, which were caused by WNV strains belonging to lineages 1 and 2, respectively. Our results identify areas of the structural proteome represented by the polypeptides clearly contains regions that were recognized preferentially by antibodies from WNV-infected individuals. The signals were heterogeneous, i.e. only a few fragments were bound strongly by most or all positive sera (e.g. peptide prM58–87). However, all WNV-positive sera showed binding over the defined background to at least one fragment, and the vast majority recognized several fragments strongly. In addition, some regions (e.g. the stretch between fragments E91–120 and E171–200 of the E-protein) displayed marginal reactivity with serum antibodies. The most antigenic linear epitopes appeared to fall within the prM/M-protein, where robust signals were detected in several fragments, most pronounced in M6–35. The capsid-protein was recognized as well, but the titters were lower compared to prM/M. Three peptides in the E-protein (E71–100, E231–260 and E171–200) displayed marginal reactivity with human sera, and E11–40 and E351–380 were not recognized at all.

| Table 1. Amino acid sequences of the fragments used in this study (lineage 1 WN strain Ita09). |
|-------------|-------------|
| Sequence    | Peptide name |
| M3KPPGPGKGSRNAVMLKFRPMPRLSVLGLG | C3–30 |
| MPRVLSLQGLRAMLSDIGGKPVRFLVAL | C21–50 |
| KGPVRFLAALLAFFRAITAAPRVALIDWRR | C41–70 |
| PTAVALDWRGWGNVKTMKHALLLSFKKGEAT | C61–90 |
| LLSFKKGEATLASSISMRRSSQQKRRGKGTG | C81–110 |
| KQKKRGKGTGAIAGMILAGSGAVTLNFIQ | C101–123/prM–1 |
| VLAGTSLNFLQGVK/WMTNTADVDVITPIPT | C121–123/prM–27 |
| DVTVDVITPIPTAAKNLCIVCAMARGVMCD | prM18–47 |
| AMDVGYMCDDTTITYCVPSLVSAGNDPEDI | prM38–67 |
| AGNDPEDIWDCWTCXTSAVYVRYGCRKTRHS | prM58–87 |
| YGRCTKRHSRISIRSLTVQTHGESTLANK | prM78–92/M–15 |
| THGESTLANKGAWMDSTKATRYLVOSTEW | MD5–35 |
| TRYLVKTESILNPNYALAVAAVGWLGS | M65–55 |
| AAVIGMVLSNMTORVFVIVLVLVPAPY | M95–75 |
| LLLVPAPYFNCLGMSNRDFLEGVSATW | M88–75/E1–20 |
| FLEGVSATWDLVLELDGQVCMSIDKDPT | E11–40 |
| VTMKSKDPTTDVKNMMeAANALAEVRSYC | E31–60 |
| ALNAEVRSYCLTVGSLDSTKACAPTMGAE | E31–80 |
| KAAAPCTMEAGHNDKRAPFADVFRCRQGVDVG | E71–100 |
| VCRQGVDVGWGNCGLFGKSDDTCACK | E91–120 |
| GSDTDCACKFASCSTKATGRTLXNKEYVA | E115–140 |
| LNKXNKEYVAIFVHGTTVSESHOXYSTIQG | E131–160 |
| SHGNYSTQIATQAGRFSIFTPAPPSTLYKL | E151–180 |
| PAAPSTLYKLGELYGEVEVDCEPRSGIDTNA | E171–200 |
| EPRSGIDTNAAYMYTGTKFLLHREVFMDO | E191–220 |
| FLYHREVFMDOHLNPWSSAGTYSWNNRETLM | E211–240 |
| TVWNRIETLMEEFEPHTAKOSVSIALGSEQE | E231–250 |
| SVIALGSEQEHALQAGAALPGIFVESFSSNTYK | E251–280 |
| PVESFSSNTVLGSLKHCRVKMLQLKGT | E271–300 |
| KEMKLGKQGTGYVCSCAFKFLGPTADGH | E291–320 |
| FLGTAPDHTGHTWVLIEQYTCDTGDPKPI | E311–340 |
| GTDGPCPKVPSVSSLTLNTPVGRLTVNP | E351–380 |
| PGVLRTTVNPFSVSTANAAYVICELLEPPFT | E351–380 |
| VLEIELAPPFDGSTVGVIRGGEQINHHWKS | E371–400 |
| QQINHHWKSGSSGIRAFFTTTTGKAQRLAA | E391–420 |
| TLGKAORLAAALGDTAWDFSGVGGVTSSVGK | E411–440 |
| VGGVFSTVGKAVHQIFGAFVRSLCGGGSWI | E431–460 |
| RSSLFGGMSWTOQQLGLALLWMGINARDRS | E461–480 |
| WMGINARDRSIALTFALAVGQLVLFLSVNH | E471–500 |
| VLLFLSVNHADTGCAIDISQRECGLCSCSGV | E491–501/N51–18 |

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or E311–340) also were recognized by many sera, albeit with lower intensity. There were no obvious differences between the samples from Italy, Greece, and the USA for the binding pattern to the different peptides or the strength of binding to individual peptides. Fragments C41–70, prM78–92/M1–15, M66–75/E1–20 and E131–160 were excluded from further analysis, as they were recognized strongly by non-immune negative sera.

We also assessed whether the heterogeneous antibody responses observed was a feature of the linear epitopes within our peptide assay. We incubated the same sera with recombinantly expressed ectodomain (residues 1–415) of the E protein (New York 1999

![Figure 1. Purification and testing of recombinant proteins. A: SDS-PAGE showing crude lysates of protein-expressing bacteria and purification steps. M, size marker; Lane 1, culture expressing GST (arrowhead); lane 2, culture expressing peptide C41–70 (asterisk); lane 3, peptide C41–70 after glutathione-affinity-purification; lane 4, peptide C41–70 after gel filtration. B: ELISA using serum I3 on GST purified only with glutathione sepharose (white column, 500 ng) or additionally with gel filtration chromatography (black column, 2000 ng). Values are the mean of two independent experiments (performed in duplicate), error bars represent the standard deviation. Statistical analysis to evaluate the difference between the two signals was performed by using an unpaired t-test, (asterisks, p<0.001). doi:10.1371/journal.pone.0066507.g001

Figure 2. Optimizing antibody binding to selected peptides. ELISA plates were coated with increasing amounts of the indicated peptides fused to GST and incubated with human sera: WNV positive sera I2, I4; G11, G12, G15 and negative serum N5. Values are the mean of two independent experiments (performed in duplicate), error bars represent the standard deviation. doi:10.1371/journal.pone.0066507.g002
Figure 3. Analysis of the binding property of selected recombinant peptides with human sera in an ELISA. 30-mer peptides spanning the WNV-proteins capsid, prM/M and E, fused to GST, were incubated with human sera from outbreaks in Italy (I1-8), Greece (G1–G15) and USA (US1-2). Those peptides displaying signals with an OD >0.5 are shown (the total number of peptides is shown in Figure S1). Values represent the absorption over cut-off (mean of four negative sera plus two standard deviations) and are derived from at least two independent experiments (performed in duplicate). Error bars represent the standard deviation. The background (binding of the serum to GST) was subtracted. doi:10.1371/journal.pone.0066507.g003
strain, lineage 1), which showed the correct tertiary structure folding, as assayed by a panel of conformational-sensitive monoclonal antibodies and X-ray crystallography [29,30]. Similar to the results obtained with the peptides, antibody titers varied substantially among the different sera (Figure 4); hence differences in IgG levels against structural proteins are intrinsic to human WNV infections and not restricted to linear peptide epitopes. Some sera (e.g. US1, G7) showed low or moderate binding to peptides spanning the E protein but bound strongly to the recombinant E-ectodomain, suggesting they recognized more complex structural epitopes, as has been described for some anti-WNV human monoclonal antibodies [31]. Although the values obtained with the two types of antigens are not directly comparable, this suggests that some individuals produce antibodies that recognize structurally discontinuous epitopes on E, which are not displayed by our peptide fragments.

To investigate the contribution of antibodies against the peptides to the overall antibody response against the E protein, competition experiments were carried out. To this end, sera were incubated with the fusion proteins displaying peptides E71–100, E231–260 or E371–400 before they were tested against the recombinant ectodomain of the E protein. GST-only was used as a control-competitor. As shown in Figure 5, a clear competition for binding was observed in some but not all sera, again highlighting the heterogeneity of the human humoral responses to WNV. In serum G1, for example, the reactivity to the E ectodomain was almost completely lost after pre-incubation with peptide E71–100, indicating that this linear epitope is a major target of the anti-E response in that particular patient. Similar results were obtained for E231–260 with serum G14 and, although less prominent, with sera G2 and G13.

**Discussion**

Information on the immune responses of humans against newly emerging European strains of WNV is still limited, especially when compared to data available after infections with North American strains. The WNV epidemic in Europe differs from that in North America in several aspects, such as pathogenicity in birds and the vector ecology [32]. Also different WNV strains from distinct genetic lineages are responsible for the outbreaks in Europe. For infections with the WNV isolates from North America, immunodominant peptides eliciting T cell responses in humans have been determined [21,33]. Although epitopes for monoclonal antibody responses have been mapped [22,29], no systematic peptide mapping has been performed with sera from infected humans. In this study, we investigated antibody responses to the structural proteins of WNV during European outbreaks, which occurred in Greece and Italy in 2010. By screening overlapping 30-mer peptides with human sera, areas in the structural proteome were identified as targets of IgG antibodies, indicating the presence of several linear B cell epitopes within these peptides.

One major observation was that the antibody profiles against the peptides corresponding to the structural proteins differed substantially between individuals. This heterogeneity of binding can best be explained by the variation in the human immune response to different epitopes and/or differences in sampling as serum was collected at different time points after symptoms onset [34]. Heterogeneity in human antibody responses against WNV

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**Figure 4. Analysis of binding properties of human sera with recombinant E ectodomain in an ELISA.** Sera used in this study were incubated with the recombinant E-ectodomain protein (lineage 1). Mean values of two independent experiments (performed in duplicate) are shown, and error bars represent the standard deviation.

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infections also was recently demonstrated by [35] who tested four different sera on recombinant WNV proteins and obtained four unique binding patterns. While differences in the HLA class I- and class II-background are associated with protection or susceptibility to neuroinvasive WNV disease [36], a direct correlation between severity of infection and the immune response to specific viral peptides could not be identified in a screen for CD8+ T cell-epitopes [21]. Analogously, we could not define a correlation between IgG responses to specific areas of the WNV-structural proteome and clinical symptoms (data not shown).

Based on the results, the immunodominant peptides were distributed throughout in the capsid (peptide C 61–90), prM/M (peptides M6–35 to M46–75), and E (peptides E71–100 and E231–260, which are in domains I and II, and E371–400 in domain III) proteins. prM/M protein was the most immunogenic in our analysis. Every infected individual in the study developed antibodies against one or several fragments of prM/M, making it a valuable protein for serologic diagnosis and thus an important constituent of VLP-based test systems [37]. The immunogenicity of prM protein in the context of human infection has been highlighted recently in studies with Dengue virus, a related flavivirus [38–40]. The region displayed by peptides M6–35 and M26–55 was shown to elicit Dengue-neutralizing antibodies in mice [41]. Within the E protein, peptides E71–100, E231–260 and E371–400 appeared immunodominant, and the three sequences were mapped onto the structure of the E ectodomain (Figure 6). Only fragment E371–400 is situated in domain III (encompassing parts of the lateral ridge FG-loop), where dominant epitopes for neutralizing antibodies in mice are found [42–44]. This data is consistent with studies suggesting that the human antibody response against E is skewed away from DIII and towards less-neutralizing epitopes in domains I and II [22,29]. Indeed, peptide E71–100, which is located near or within the fusion loop in domain II of the E protein, was one of the dominant peptides in the present analysis; however, fusion loop reactive monoclonal antibodies tend to have poorly neutralizing activity against lineage 1 strains of WNV [45,46].

The results obtained with the binding to individual peptides were confirmed by competition studies between peptides and the recombinant E-ectodomain. In some sera (G1, G2, G15), which showed strong binding to only one peptide in the E-protein (Figure 3), the signal for the E-ectodomain was reduced after pre-incubation with this peptide. As G1, G2 and G15 recognize the folded E-ectodomain well (Figure 4), these results indicate that the linear sequences corresponding to E71–100, E231–260 and E371–400 are accessible for antibody binding. Using sera that showed binding to multiple E-peptides in the screen, the competition with a single peptide did not significantly change binding to the E-ectodomain (e.g. G8, G11 or G12). However, serum G4 only binds strongly to E71–100, yet there was no competition with this peptide. Together with the finding that in sera G2, G14 and G15 there was still a considerable signal left after competition, the data from the competition tests confirm that antibodies against tertiary structural epitopes on the E-ectodomain, not displayed by our peptides, are generated preferentially during infection in some individuals.

By using sera from a WNV outbreak in Israel, [47] described a linear B-cell epitope in the E protein, which reacted with some, but not all WNV positive sera. That epitope, located within E351–380, only was recognized marginally by the sera from our study. Differences in the infecting strain (e.g. the Ita09 strain contains the point mutation A369S within the epitope), in the B cell response by different populations, or in the methodology (e.g. the EP15 epitope of [47] was used as a tandem peptide fused to a phage protein) might contribute to the disparity in findings.

Somewhat surprisingly, all humans did not develop robust antibody responses against the recombinant E protein. Serum I3
Color. DI, DII and DIII indicate the respective domains.

had low levels of antibodies against the E protein ectodomain or peptides, although a clear response against prM peptide M₆–₃₅ was observed. One caveat to this analysis is that antibodies by patient 13 could have been generated against E protein epitopes that are present exclusively on the icosahedral surface of the intact virion, which are absent from isolated recombinant E protein or peptides. Indeed, such quaternary epitopes that localize to the hinge region between domains I and II on the E protein have been described for human monoclonal antibodies against WNV and DENV [31,49–50]. Antibodies against the capsid mainly reflect immune responses against disrupted virions or apoptotic or necrotic infected cells in which intracellular contents are released. These are unlikely to have a protective phenotype, except supporting antigen presentation by antigen presenting cells.

In summary, we show that several peptides of the capsid, the prM/M, and the E proteins of WNV are recognized by IgG-antibodies from humans infected with newly emerging European strains of WNV. All WNV-positive sera bound strongly to at least one peptide (corresponding to the lineage 1 amino acid sequence) and no clear differences were observed between sera obtained from patients from Greece, Italy or the USA that were infected with lineage 2 or 1 strains. However, the data reveal substantial differences between individual sera in the patterns of the proteins and domains recognized, highlighting the heterogeneity of the human humoral immune response against WNV, which should be considered during the development of specific diagnostic tests.

Materials and Methods

Protein Expression and Purification

The sequence coding for amino acids 1–810 of the lineage 1 WNV strain “Ita09” (NCBI Acc#. GU011992) was separated into 40 clones, each coding for 30 amino acid long peptides with an overlap of 10 amino acids on both sides. The length of overlapping 30 amino acids was chosen to optimally detect long linear epitopes in combination with fine-mapping of the humoral response. The DNA fragments were produced in the bacterial expression vector pEXP1 by ATG: biosynthetics (Freiburg, Germany) and contain DNA fragments were produced in the bacterial expression vector pEXP1 by ATG: biosynthetics (Freiburg, Germany) and contain

Figure 6. Structure of the E-ectodomain of WNV. The sequences corresponding to the peptides E71–100, E231–260 and E371–400 are highlighted in color. DI, DII and DIII indicate the respective domains.
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Sera

Serum samples from confirmed WNV-infections were obtained during outbreaks in Italy and Greece in 2010. The Italian samples (University of Padova, Italy) were derived from seroprevalence studies, blood donors or patients with West Nile neuroinvasive disease. The Greek samples (University of Thessaloniki, Greece) were from patients with neuroinvasive disease, taken during the acute phase of illness (3–17 days). In addition, two samples were obtained from Seracare (USA). None of the patients were vaccinated against other flaviviruses or had a recent travel history to other countries endemic for WNV. Patients were 32–83 years old, and only one was born before the Dengue-outbreak in Greece in 1927 [18]. WNV-negative samples were from each source and from the University of Leipzig (Germany). The study was approved by the Padova University Hospital ethics committee and all persons provided written consent.

Antibody – Peptide Binding Analysis by ELISA

Nunc polysorb plates (Thermo Scientific, Germany) were coated in duplicate with 2 micrograms of GST-fusion proteins or with 100 nanograms of recombinant E ectodomain protein (in coating buffer [15 mM Na₂CO₃, 35 mM NaHCO₃ pH 9.6]) per well and were incubated over night at 4°C with gentle agitation. The plates were then washed three times with 350 microliters per well of PBS/Tween (0.05%), followed by blocking with 5% non-fat dry milk powder (200 microliters per well) for 2 h at room
temperature (RT). After a second wash step, the human sera (dilution 1:100 in 5% non-fat dry milk powder, 100 microliters per well) were incubated for 1.5 h at RT. The sera were removed by a third wash step and 100 microliters of the secondary antibody (1:5000 diluted HRP-conjugated Goat-anti-Human IgG, Fisher Scientific) was added for 1 h at RT. After washing, the TMB-substrate (BioLegend, Germany) was added to the wells and the plate was incubated for 30 min at RT in darkness. To stop the reaction, 1 M H$_2$SO$_4$ was added, followed by measurement at 450 nm and 520 nm (reference wavelength) in an ELISA Reader.

The cut-off-values were defined for each individual antigen by calculating the mean of four negative sera (derived from Italy, Greece and Germany) and adding two standard deviations. Competition ELISA was performed as described above, except that 50 nanograms recombinant E ectodomain was coated and the sera were pre-incubated with 4 micrograms GST-fusion protein or GST for 45 min at RT.

**Supporting Information**

**Figure S1** Analysis of the binding property of all recombinant peptides used in the study with human sera in an ELISA. 30-mer peptides spanning the WNV-proteins capsid, prM/M and E and a small part of NS1, fused to GST, were incubated with human sera from outbreaks in Italy (I1-8), Greece (G1-G15) and USA (US1-2). Values represent the absorption over cut-off (mean of four negative sera plus two standard deviations, indicated below the peptide names) and are derived from at least two independent experiments (performed in duplicate). Error bars represent the standard deviation. The background (binding of the serum to GST) was subtracted. (PDF)

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**Author Contributions**

Conceived and designed the experiments: SU SC. Performed the experiments: SC AK JMR. Analyzed the data: SC AK GRM MSD SU. Contributed reagents/materials/analysis tools: GP LB AP PF UGL MN. Wrote the paper: SC SU. Critically revised the manuscript for important intellectual content: GP LB AP PF UGL MSD.

**References**

1. Hayes EB, Seijar JJ, Zaki SR, Lanciotti RS, Bode AV, et al. (2005) Virolology, pathobiology, and clinical manifestations of West Nile virus disease. Emerg Infect Dis 11: 1174–1179.

2. Murray KO, Mertens E, Despres P (2010) West Nile virus and its emergence in the United States of America. Vet Res 4: 67.

3. Barzon L, Franchin E, Squarzon L, Lavezzo E, Toppo S, et al. (2009) Genomic sequence analysis of the first human West Nile virus isolated in Italy. 2009. Euro Surveill 14.

4. Serbu A, Cizauskaite CS, Pancalèse-Gatji RJ, Vázquez A, Tenorio A, et al. (2010) Outbreak of West Nile virus infection in humans, Romania, July to October 2010. Euro Surveill 2011;16

5. Barzon L, Pacenti M, Franchin E, Lavezzo E, Martello T, et al. (2012) New endemic West Nile virus lineage 1a in northern Italy, July 2012. Euro Surveill 17.

6. Papa A (2012) West Nile virus infections in Greece: an update. Expert Rev Anti Infect Ther 10: 743–750.

7. Danni K, Papa A, Theocharopoulos G, Deugas G, Athanasiou M, et al. (2011) Outbreak of West Nile virus infection in Greece, 2010. Emerg Infect Dis 17: 1667–1672.

8. Gubler DJ, Kuno G, Markoff L (2007) Flaviviruses. In: Knipe DM, Howley PM. **Viral Pathogenesis and Vaccinology** (ed. Jayne H. B. and Howley P. M.). Lippincott Williams and Wilkins: 1153–1252.

9. McCollough B, Suh RJ, Rossman MG (2005) A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3: 13–22.

10. Lanciotti RS, Ebel GD, Deubel V, Kestler AJ, Murr S, et al. (2002) Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 298: 96–105.

11. Barzon L, Pacenti M, Franchin E, Squarzon L, Lavezzo E, et al. (2012) Novel West Nile virus lineage 1a full genome sequences from human cases of infection in north-eastern Italy, 2011. Clin Microbiol Infect 18: E341–544.

12. Savini G, Capelli G, Monaco F, Polci A, Russo F, et al. (2012) Evidence of West Nile virus lineage 2 circulation in Northern Italy. Vet Microbiol 17: 267–73.

13. Magurano F, Remoli ME, Baggieri M, Fortuna C, Marchi A, et al. (2012) Novel West Nile virus lineage 2 circulation in Northern Italy. Vet Microbiol 17: 267–73.

14. Upton RM, Meade X, Meade S, Meade A, et al. (2012) West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. Clin Microbiol Infect 18: E545–547.

15. Diamond MS, Muhlhöpp E, Oliphant T, Samuel MA (2009) The host immunologic response to West Nile encephalitis virus. Front Biosci 14: 3024–3034.

16. Diamond MS, Shrestha B, Marri A, Mahan D, Engle M (2003) B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. J Virol 77: 2578–2586.

17. Diamond MS, Siut J, Freund L, Higgs S, Shrestha B, et al. (2003) A critical role for induced IgM in the protection against West Nile virus infection. J Exp Med 190: 1853–1862.

18. Papa A, Karabashovou D, Konsouzidou A (2011). Acute West Nile virus neuroinvasive infections: cross-reactivity with dengue virus and tick-borne encephalitis viruses. J Med Virol 83: 1861–1865.
35. Faggioni G, Pomponi A, De Santis R, Masuelli L, Ciammaruconi A, et al. (2012) West Nile alternative open reading frame (N-NS4B/WARF4) is produced in infected West Nile Virus (WNV) cells and induces humoral response in WNV infected individuals. Virol J 9: 283

36. Lanteri MC, Kitzlerova Z, Peterson T, Cate S, Guster B, et al. (2011) Association between HLA class I and class II alleles and the outcome of West Nile virus infection: an exploratory study. PLoS One 6: e22946.

37. Davis BS, Chang GJ, Cropp B, Roehrig JT, Martin DA, et al. (2001) West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 75: 4040–4047.

38. Dejnirattisai W, Jumnainsong A, Otsirirakul N, Finton P, Vasanawathana S, et al. (2010) Cross-reacting antibodies enhance dengue virus infection in humans. Science 328: 745–748.

39. Beltramello M, Williams KL, Simmons CP, Macagno A, Simonelli L, et al. (2016) The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe 8: 271–283.

40. de Alwis R, Beltramello M, Messer WB, Sukupolvi-Petty S, Wahala WM, et al. (2011) In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection. PLoS Negl Trop Dis 5: e1198.

41. Vázquez S, Guzmán MG, Guilleen G, Chinea G, Pérez AB, et al. (2002) Immune response to synthetic peptides of dengue prM protein. Vaccine 20: 1823–1830.

42. Diamond MS, Pierson TC, Fremont DH (2006) The structural immunology of antibody protection against West Nile virus. Immunol Rev. 2008 Oct;225: 212–225.

43. Beasley DW, Barrett AD (2002) Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol 76: 13097–13100.

44. Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nature Med. 11: 522–530.

45. Oliphant T, Nybakken GE, Engle M, Xu Q, Nelson CA, et al. (2006) Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. J Virol 80: 12149–12159.

46. Vogt MR, Dowd KA, Engle M, Teoh RB, Johnson S, et al. (2001) Poorly neutralizing cross-reactive antibodies against the fusion loop of West Nile virus envelope protein protect in vivo via Fcgamma receptor and complement-dependent effector mechanisms. J Virol 85: 11367–11370.

47. Herrmann S, Leshem B, Lobel I, Bin H, Mendelson E, et al. (2007) T7 phage display of Ep13 peptide for the detection of WNV IgG. J Virol Methods 141: 135–140.

48. Kaufmann B, Vogt MR, Goudsmidt J, Holdaway HA, Aksyuk AA, et al. (2010) Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. Proc Natl Acad Sci U S A 107: 18950–18955.

49. de Alwis R, Smith SA, Olivarez NP, Messer WB, Huyhn JP, et al. (2012) Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. Proc Natl Acad Sci U S A 109: 7439–7444.

50. Teoh EP, Kukkaro P, Teo EW, Lim AP, Tan TT, et al. (2012) The structural basis for serotype-specific neutralization of dengue virus by a human antibody. Sci Transl Med 4: 139ra83.