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Research paper

Antigenic relationship among zoonotic flaviviruses from Italy

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ABSTRACT

Here we report studies of the antigenic relationship of West Nile virus (WNV) and Usutu virus (USUV), two zoonotic flaviviruses from Italy, together with a Japanese encephalitis virus (JEV) strain and compared them with their genetic relationship using the immunodominant viral E protein. Thirty-nine isolates and reference strains were inactivated and used to immunize rabbits to produce hyper immune sera. Serum samples were tested by neutralization against all isolates and results visualized by generating antigenic map. Strains of WNV, USUV, and JEV grouped in separate clusters on the antigenic map. JEV was closer antigenically to USUV (mean of 3.5 Antigenic Unit, AU, equivalent to a 2-fold change in antibody titer) than to WNV strains (mean of 6 AU). A linear regression model predicted, on average, one unit of antigenic change, equivalent to a 2-fold change in antibody titer, for every 22 amino acid substitutions in the E protein ectodomain. Overall, antigenic map was demonstrated to be robust and consistent with phylogeny of the E protein. Indeed, the map provided a reliable means of visualizing and quantifying the relationship between these flaviviruses. Further antigenic analyses employing representative strains of extant serocomplexes are currently underway. This will provide a more in deep knowledge of antigenic relationships between flaviviruses.

1. Introduction

Zoonotic mosquito-transmitted flaviviruses (genus *Flavivirus*, family *Flaviviridae*) constitute one of the main challenges for human and animal health. Dengue viruses (DENV) cause around 21.000 human deaths annually, and it is estimated that at least 120 countries have endemic DENV transmission (Thomas and Endy, 2011); In affected areas, Zika virus (ZIKAV) was recently shown to be responsible for microcephaly and other severe brain defects following infections of pregnant women (Chibueze et al., 2017). West Nile virus (WNV) has become more prominent as a zoonotic agent, particularly in Europe, where infections in humans, horses and birds have been reported. Humans can develop disease which may range from mild febrile illness (West Nile fever) to encephalitis with fatal outcome (Kramer et al., 2007). Usutu virus (USUV) is a close relative of WNV but was considered for decades as a flavivirus with low zoonotic potential. The first large outbreak of USUV occurred in 2001, in Austria, with a significant die-off of Eurasian blackbirds (*Turdus merula*) and great grey owls (*Strix nebulosa*) (Weissenböck et al., 2002). Recent data from various European countries indicate that there also might be a much higher number of clinical neuro-invasive USUV infections in humans than had been assumed to date (Grottola et al., 2017; Cadar et al., 2017). Both viruses circulate worldwide in multiple genetic lineages (L) (Cadar et al., 2017; Engel et al., 2016; Bakonyi et al., 2017). According to recent surveillance, WNV and USUV are the most widespread mosquito-borne flaviviruses in Italy, with co-circulation in the same geographical areas (Mancini et al., 2017; Victoriano Llopis et al., 2015; Grottola et al., 2017).

Viruses in the genus *Flavivirus* are placed into complexes with shared antigenic cross-reactivity. WNV and USUV belong to the Japanese encephalitis virus (JEV) serocomplex along with JEV, Murray Valley encephalitis virus and St. Louis encephalitis virus. Other serocomplexes include yellow fever virus, DENV and tick-borne encephalitis virus, which contains three subtypes and louping ill virus (Porterfield, 1980). This classification has largely been supported by genomic phylogeny, but the correspondence between genetic and phenotypic expression is not clear. A previous study demonstrated complex antigenic relationships among the flaviviruses, but used sera

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from individuals that had been vaccinated against more than one virus, complicating interpretation (Mansfield et al., 2011). Thus, flaviviruses constitute a challenge for serological diagnosis and prediction of vaccine cross-protection. Indeed, specificity in flavivirus antibody-measurement is significantly hampered by the structural similarity of the viruses and the resulting antibody cross-reactivity. The antigenic characteristics of flaviviruses are mainly determined by their envelope (E) protein structures (Heinz and Stiasny, 2017). This protein is widely used in serological diagnosis, as virtually all infected individuals generate quantifiable IgG antibodies against it. The E is an approximately 53 kD type I membrane glycoprotein. It has three domains (D1-D3). D1 is the N-terminal structurally central domain while D2 is the dimerization domain. The D3 functions in receptor recognition and binding (reviewed in Iyer and Kousoulas, 2013). Resistance of WNV to antibody-mediated neutralization has been directly correlated with mutations in the epitopes of D3 (Maillard et al., 2008), and a potential role for D3 in cross-protection among flaviviruses has been suggested by results of studies in mice (Li et al., 2013). Serological cross reaction has been demonstrate to occur also between WNV and USUV (Blázquez et al., 2015).

The analysis of antigenic differences within circulating viruses, with the notable exception of influenza viruses (Bedford et al., 2014) due to the pressing need for accurate vaccine strain selection, is not as timely or accurate as the genetic characterization. Despite close correspondence between genetic and antigenic variation, significant differences are observed. In influenza A virus genetic change may have a disproportionately large antigenic effect. Even one single nucleotide (nt) mutation, may have important consequences for antigenicity and receptor binding avidity (Li et al., 2013; Koel et al., 2013; Lorusso et al., 2014). To visualize and quantify the antigenic relationships among influenza strains, antigenic cartography was assessed for the first time fifteen years ago for H3N2 strains isolated from 1968 to 2003 (Smith et al., 2004). During the last decade, this method was used to measure the antigenic distance of influenza viruses (Smith et al., 2004; de Jong et al., 2007; Russell et al., 2008; Garten et al., 2009; Lorusso et al., 2011), lyssaviruses (Horton et al., 2010), enteroviruses (Huang et al., 2009), picornaviruses (Ludi et al., 2014) and flaviviruses (Mansfield et al., 2011). In this manuscript, we showed the antigenic relationships for a set of 33 strains of zoonotic flaviviruses (WNV and USUV) isolated in Italy in the last ten years, and compared with their genetic relatdness.

2. Materials and methods

2.1. Viruses

A total number of 25 strains including 17 WNV L1 and 8 WNV L2 isolated in Italy from different hosts and geographical areas were employed for the analysis (Table 1). These strains are stored at the Italian Reference Laboratory for Exotic Diseases (CESME) of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (IZSAM). Two WNV L1 and one WNV L2 (Eg101, NY99 and B956, respectively) reference strains were also added to the analysis. Moreover, eight USUV strains isolated from birds in Italy were also included. Likewise, two USUV reference strains (939 and SAAR of the European and African lineages, respectively) were also added. One JEV isolate was also used for the analysis. Italian field strains used in this study represent all the isolates obtained between 2008 and 2015 within the framework activities of the National surveillance plan for WNV. Isolation was performed following procedures previously described (Savini et al., 2013).

2.2. Ethics

All procedures on animals were accomplished respecting the European and Italian regulations on the use of animals for experimental purposes. The study was approved by the Animal Welfare Committee of IZSAM and authorized by the Italian Ministry of Health (authorization number 48/2015-PR).

2.3. Inactivation and production of hyperimmune sera

Low cell-passages (p4, onto African green monkey kidney cells-Vero) of field strains were used for inactivation. Viruses for immunization were prepared at approximately 10^6 tissue culture infective doses- $TCID_{50}$/ml per 20 ml, inactivated by binary ethylenimine (BEI, 5 mM; Ronchi et al., 2012). A commercial adjuvant (Montanide™ Gel, Seppic Srl, Milan-Italy) was then added at a ratio of 1: 1 (*v*/v) virus to adjuvant. Inactivation was verified by propagating each virus onto Vero cells for three times followed by immunofluorescence. Adult New Zealand white female and male rabbits (of nearly 3.0 kg of weight) were employed for production of hyperimmune serum. Rabbits were housed in wire cages (one individual per cage) under controlled environmental conditions with free access to food and water. Prior immunization, blood was collected from all animals in EDTA tubes and recovered plasma was screened to confirm the absence of WNV/USUV antibodies by cELISA (ID Screen West Nile Competition Multi-species, IDvet-Grabels, France). Rabbits were administered following sedation (Prequillan®, Fatro) and general anesthesia (Zoletil®, Virbac® + Rompun®, Bayer) with the inactivated viruses. Two rabbits per virus were immunized at day (d) 0 and d2 with inactivated virus combined with commercial adjuvant (1 ml in total) by intramuscular (IM) and intraperitoneal (IP) routes. At d4, in addition to the IM administration, inactivated virus and adjuvant (1 ml in total) were also given by a subcutaneous route. At d6, inactivated virus and adjuvant (3 ml in total) were administered by IM with four different injections of 0.75 ml each in the legs and shoulders. At d8, the same protocol of d6 was used. At d21, serological screening by serum-neutralization (SN) with the homologous strain was adopted to confirm the seroconversion of immunized animals. At the end of the vaccination period, rabbits were humanely euthanized with Tanax® (Intervet).

2.4. Serological assay

Sera were heat inactivated at 56 °C. Serum samples from rabbits were tested by SN (Di Gennaro et al., 2014) against all WNV, USUV and JEV isolates used for immunization. Briefly, starting from a dilution titer of 1:5, serial 2-fold dilutions were made in microtiter plates, and 100 tissue culture infective doses (TCID) of antigen were added to each dilution. Thereafter, the mixtures were incubated at 37 °C for 1 h, and 10^5 Vero cells were added to each well. The plates were incubated at 37 °C for 5 days. Starting from the third day after incubation, the plates were checked for cytopathic effect, and the antibody titer was defined as the reciprocal of the highest dilution of the serum that showed 100% neutralization. The reciprocal of the ratio between heterologous and homologous SN titers for individual serum samples was calculated to determine the fold difference between heterologous and homologous reactions. SN plates were blinded to prevent bias.

2.5. Characterization of the E protein

As the E protein is the principal antigen responsible for eliciting a neutralizing antibody response, field strains were also sequenced by Next Generation Sequencing (NGS, Marcacci et al., 2016) or standard RT-PCR protocols. The amino acid sequences of the E protein of the tested strains were compared. Whole genome characterization and epidemiological analysis of circulating field strains are currently underway (Monaco et al., manuscript in preparation).

2.6. Phylogeny

E protein sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy (MUSCLE with default settings). The analysis

Table 1
Relevant data regarding the isolates used for the present study.

Strain number	Viral species	Isolation material	Host	Municipality	Province	Year of isolation
15076	WNV L1	Organs pool	Magpie	Ferrara	Ferrara	2008
15217	WNV L1	Organs pool	Magpie	Jolanda di Savoia	Ferrara	2008
15242	WNV L1	Plasma	Donkey	Tresigallo	Ferrara	2008
15802	WNV L1	Organs pool	Pigeon	Voghera	Pavia	2008
15803	WNV L1	Organs pool	Magpie	Voghera	Pavia	2008
15325	WNV L1	Plasma	Horse	Trecenta	Rovigo	2008
15250	WNV L1	Brain	Horse	Ferrara	Ferrara	2008
12010	WNV L1	Organs pool	Magpie	Luzzara	Reggio Emilia	2009
19,883	USUV	Hearth	Crow	Chioggia	Venezia	2009
12543	USUV	Organs pool	Blackbird	Urbino	Pesaro-Urbino	2010
12628	USUV	Organs pool	Blackbird	Treia	Macerata	2010
12962/4	USUV	Hearth	Blackbird	Senigallia	Ancona	2010
12962/7	USUV	Kidney	Blackbird	Senigallia	Ancona	2010
12963/2	USUV	Spleen	Blackbird	Senigallia	Ancona	2010
12963/4	USUV	Kidney	Blackbird	Osimo	Ancona	2010
20224/1	WNV L1	Plasma	Chicken	Santa Giusta	Oristano	2011
20224/8	WNV L1	Plasma	Chicken	Santa Giusta	Oristano	2011
21412	WNV L1	Brain	Owl	Oristano	Oristano	2011
23237/1	WNV L1	Plasma	Chicken	Arborea	Oristano	2011
14444	WNV L1	Organs pool	Magpie	Ro	Ferrara	2011
17196	WNV L1	Organs pool	Owl	Bondeno	Ferrara	2011
17208	WNV L1	Organs pool	Crow	Gaggio Montano	Bologna	2011
21370	WNV L1	Plasma	Horse	Mogoro	Oristano	2011
20168	WNV L2	Brain	Goshawk	Usellus	Oristano	2012
20652	WNV L1	Organs pool	Magpie	Latisana	Udine	2012
19882	USUV	Organs pool	Crow	Olbia	Olbia-Tempio	2012
27449	WNV L2	Brain	Goshawk	Muros	Sassari	2013
19685	WNV L2	Organs pool	Magpie	Cavezzo	Modena	2015
21926	WNV L2	Kidney	Real owl	Balzola	Alessandria	2015
21863	WNV L2	Organs pool	Magpie	San Cesario sul Panaro	Modena	2015
21864	WNV L2	Organs pool	Magpie	San Cesario sul Panaro	Modena	2015
22115	WNV L2	Insects	*Culex pipiens*	Ticineto	Alessandria	2015
21865	WNV L2	Organs pool	Magpie	Castelfranco Emilia	Modena	2015
			Acc nos		**Country and city**	
Eg101	WNV L1		AF260968		Egypt	1951
NY99	WNV L1		AF202541		USA, New York	1999
B956	WNV L2		NC001563		Uganda	1937
USUV 939	USUV		AY453411		Austria,Wien	2001
USUV SAAR	USUV		AY453412		South Africa	1959
JEV Lederle	JEV	Lederle Laboratories				

was performed on the Phylogeny.fr platform (http://www.phylogeny.fr/index.cgi). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.055) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 1.649). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

2.7. Antigenic cartography

Antigenic relationships between viruses were quantified and visualized by generating antigenic maps as previously described (Mansfield et al., 2011; Smith et al., 2004). Maps were made by calculating a target distance between each virus and antiserum from the neutralization titers and positioning the virus and antiserum on a map such that the distance between them was as close as possible to the target distance (minimizing the sum-squared error between map distance and target distance). The target distance was calculated as the difference between the logarithm (log_2) reciprocal neutralization titer for that particular virus and the log_2 reciprocal maximum titer achieved by that serum (against any virus), so the higher the titer, the lower the target distance. Antigenic cartography, implemented using ACMACS (www.antigenic-cartography.org)* was then used to optimize the positions of the viruses and sera relative to each other on a map. Multiple maps were made in two, three and four dimensions, by 100 random-restart optimizations, ranked in order of total error and quantitatively compared to each other, for self-consistency. Three dimensions was chosen as the most appropriate to visualize the antigenic relationships based on these analyses. More details are available in Supplemental Material.

3. Results

3.1. Serological assay

All obtained serum samples were tested against all isolates. Results of homologous (highlighted in yellow boxes) and heterologous reactions are available in Supplementary Material. In our setting, isolates representing the WNV species, regardless of the lineage or the year of isolation, demonstrated from null cross-reactivity to strong cross-reactivity (> 1:640). Similar scenario was also revealed for USUV strains. Cross-reactivity between WNV and USUV strains was variable, ranging from null cross-reactivity to very low cross-reactivity (1:10). The lack of cross-reactivity was demonstrated between WNV strains and JEV whereas cross-reactivity between JEV and USUV ranged from no cross-reactivity to moderate cross-reactivity (1:80).

3.2. Antigenic relationships

USUV, WNV and JEV strains used in this study were demonstrated to be in separate clusters on the antigenic map (Fig. 1).The total

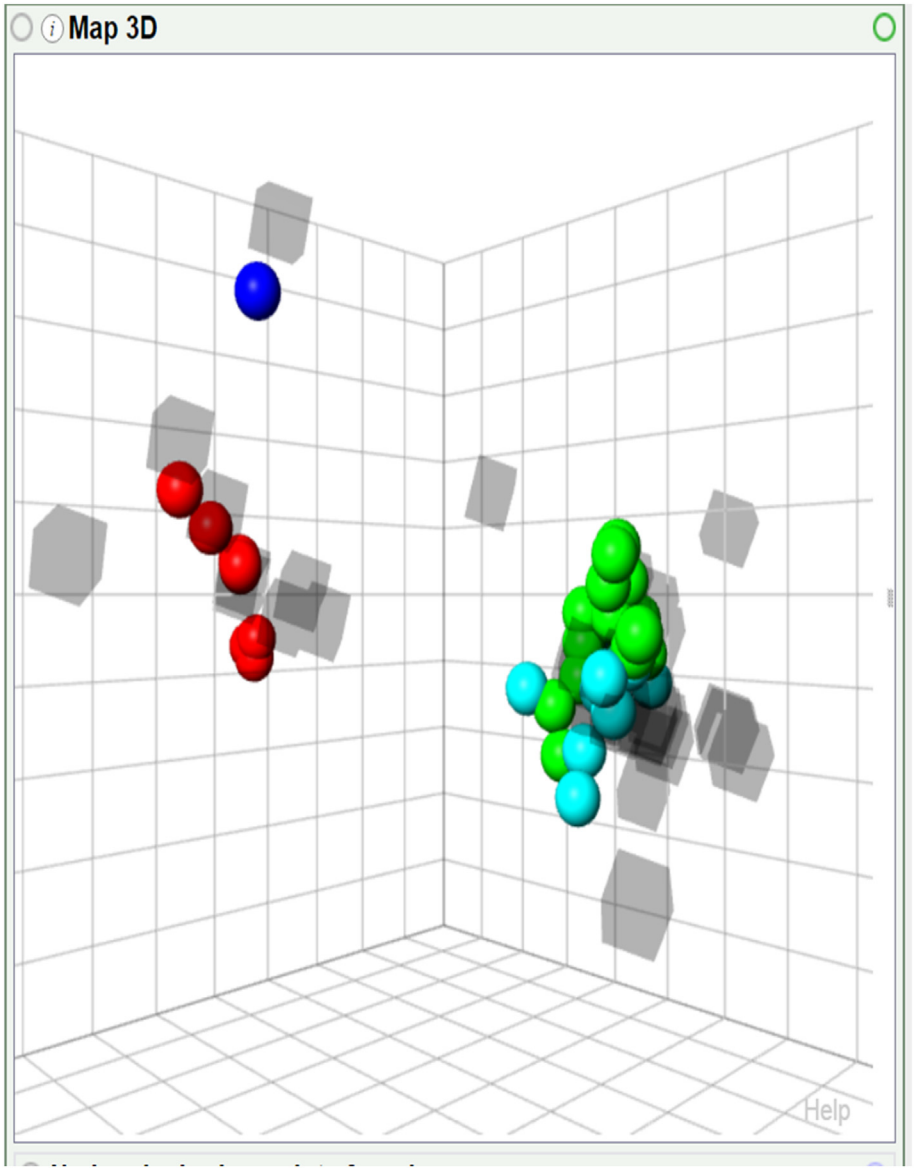

Fig. 1. 3D antigenic map of representative historical and contemporary WNV and USUV strains. Red dots, USUV; Green dots, WNV L1; Light blue dots, WNV L2; Blue dot, JEV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antigenic variation among the representatives of USUV (three antigenic units, AU) was similar to the variation among the WNV isolates. Strains from WNV L1 were, however, not antigenically distinguishable from L2. Although they belong to the same serogroup, JEV was closer antigenically to the USUV (mean of 3.5 AU) than the WNV strains (mean of 6 AU). The antigenic proximity of JEV with USUV concurred with the phylogeny of the E protein (Fig. 2). The apparent clustering of sera (grey cubes) together with colored dots (viruses) in the 3D map confirms that they were hyperimmune sera specifically produced for the selected viruses. However, in few cases cross-reaction was revealed between WNV and USUV and between USUV and JEV. Antigenic maps were consistent, and robust to addition/removal of viruses. Thus, antigenic cartography provided, in this case, a reliable means of visualizing and quantifying the relationship between the selected flaviviruses.

3.3. Analysis of the E protein

An overall aa identity of the E protein ranging from 99.4 to 100% was demonstrated within WNV L1 strains, from 99.7% to 100% within WNV L2 strains, 93.3% to 95.6% between WNV L1 and L2 strains, 76.2% to 78.0% between WNV (regardless of the lineage) and USUV strains. Italian USUV field strains were nearly identical within each other. By employing one representative isolate for each viral genotype (Fig. 3), aa relatedness with JEV E protein was 76%, 77% and 78% for WNV L1, L2 and USUV, respectively. However, aa identity in the D3 domain was higher (84%) between JEV and USUV with respect to that observed between homologous domains of JEV with WNV L1 (77%) and WNV L2 (76%). Aa identities between homologous D1 and D2 of USUV and JEV were 73% and 77%, respectively.

3.4. Predicting antigenic distance from amino acid sequence homology

Antigenic distances measured from the antigenic map were compared with differences from an *E*-protein amino acid similarity matrix. Each virus was compared with every other virus, giving an estimate of the correlation between antigenic and amino acid distances. Pairwise comparison of all antigenic distances with amino acid distances showed a statistically significant correlation ($r = 0.91$ (Fig. 4). In addition, a linear regression model predicts on average, one unit of antigenic

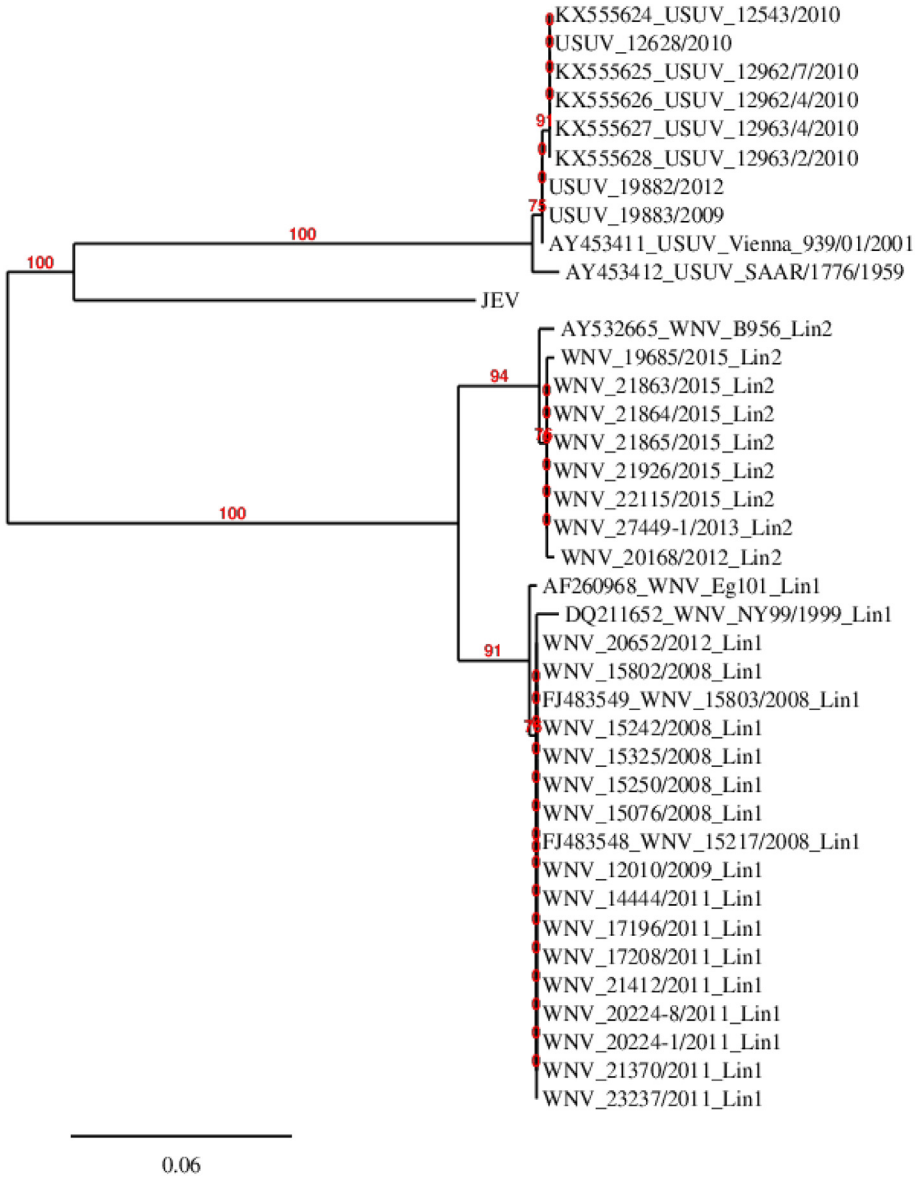

Fig. 2. Phylogeny of the E protein of the flaviviruses used in the study. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). Boostrap values ≥70 are showed. Bar indicates the estimated numbers of aa substitutions per site. The analysis was performed on the Phylogeny.fr platform (http://www.phylogeny.fr/index.cgi).

change, equivalent to a 2-fold change in antibody titer, for every 22 amino acid substitutions in the *E*-protein ectodomain.

4. Discussion

In this study, we investigated the antigenic relationships between a number of field WNV and USUV strains which originated from biological samples collected between 2008 and 2015 during the activities of an integrated serological, entomological and virological surveillance program for West Nile neuroinvasive disease implemented at the national level by the Italian Ministry of Health. While isolates used for cartography were representative of both WNV L1 and L2, which are the unique WNV lineages circulating in Italy and with distinct genomic signatures, USUV field strains were all nearly identical to each other as they belonged to the main European lineage. Reference WNV and USUV isolates and one JEV isolate were also included in the analysis. Whereas reference WNV strains including Eg101, NY99 and B956 belong to L1 and L2, respectively, USUV strain 1776 isolated in the Republic of South Africa in 1959 is representative of the Africa 2 lineage (Cadar et al., 2017).

The antigenic relationships were depicted in a 3D map. This map was created by interpreting serum-neutralization results. The map was demonstrated to be robust and consistent with phylogeny of the E protein. The map provided a reliable means of visualizing and quantifying the relationship between these flaviviruses. JEV was demonstrated to be antigenically closer to USUV than WNV as indicated also by phylogeny of the E protein. The highest % of aa sequence identity between JEV and USUV strains was demonstrated in the D3 domain of the E protein. As this domain has been indicated to be involved in cross-protection among flaviviruses, it is tempting to speculate that the proximity of these viruses in the antigenic map is related to the observed identity in this domain. Intriguingly, although cross reaction between JEV and WNV was not revealed in our experimental settings, previous studies demonstrated cross reaction between JEV and WNV *in vitro*, albeit with sera from individuals vaccinated against multiple agents (Mansfield et al., 2011) and *in vivo* with a prominent role of D3 as inducer of protective antibody response in mice challenged with a virulent WNV strain (Li et al., 2011). Nevertheless, antigenic

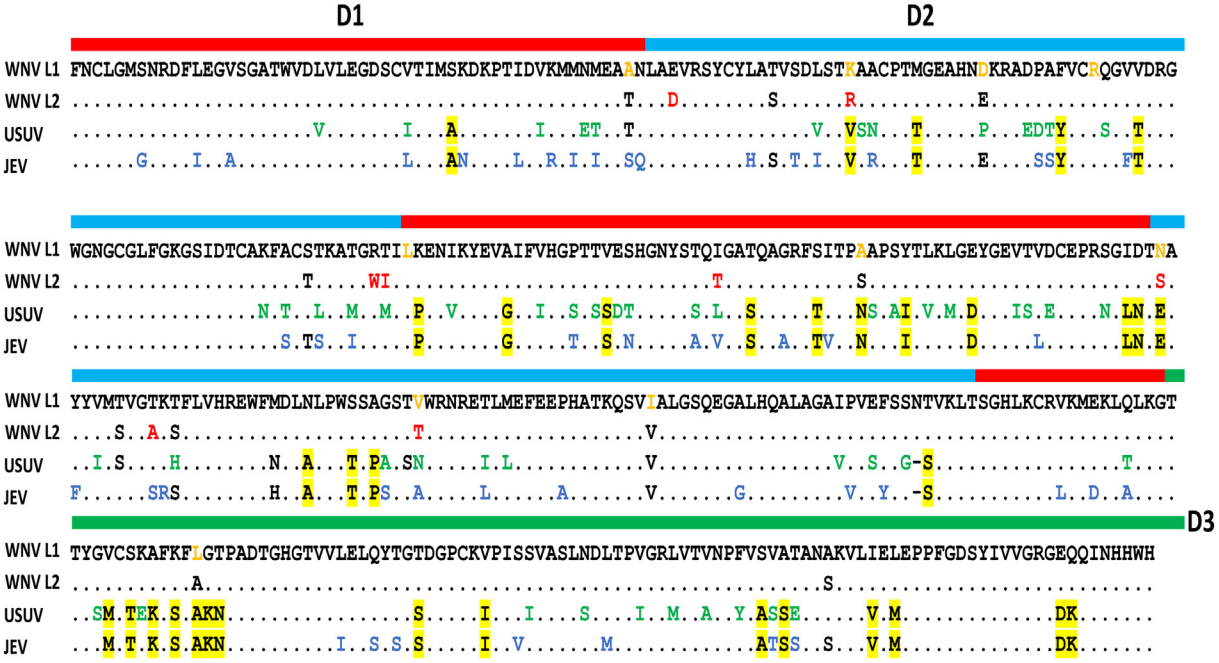

Fig. 3. Alignment of the E proteins. One selected strain per viral genotye has been used. Red, D1 domain; Blue, D2 domain; Green, D3 domain. Aa shared by USUV and JEV are highlighted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

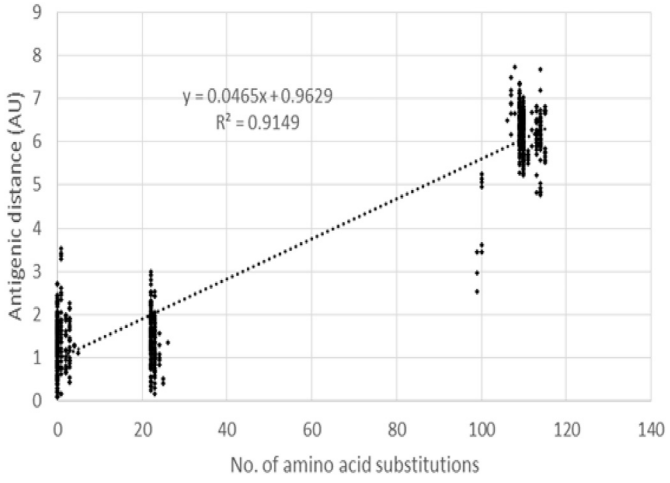

Fig. 4. Plot of pairwise antigenic and genetic distances between viruses. Antigenic distances are measured from the antigenic map, in antigenic units. Genetic distances between viruses are represented as the number of amino acid substitutions in the E protein ectodomain. The line represents a linear regression model ($R^2 = 0.91$), which predicts, on average, an antigenic distance change of one antigenic unit, equivalent to a 2-fold change in neutralization titer, per 22 amino acid substitutions.

cartography, which was previous applied to other pathogens, allowed high resolution quantitative analyses and visualizations of antigenic relationships between the tested field viruses. The viruses clearly grouped in three main clusters and strains of WNV L1 were not antigenically distinguishable from WNV L2 strains. One could reasonably point out that in a few cases, no cross-reactivity was evidenced between some WNV strains. However, because antisera were tested against multiple antigens, and antigens tested against multiple antisera, many measurements were used to determine the position of the antigen and antiserum points in an antigenic map, thus increasing the accuracy of point placement beyond that of individual SN measurements. One advantage of antigenic cartography is that it is minimally dependent on individual variations in serological response compared with other interpretations of antigenic data. Indeed, assays of the same strain and antiserum can occasionally produce different values, as it is the case of this study, even in the same laboratory. Accordingly, although cross reactions between WNV and USUV and between USUV and JEV were observed in a few cases, they did not cluster together in the map.

This study clearly has some limitations. First, SN data were obtained by using hyper-immune sera raised against whole inactivated viruses, and not purified E proteins; these sera may contain, therefore, neutralizing antibodies for other viral proteins. However, the E protein is considered the major antigen of flaviviruses inducing neutralizing Ab in infected individuals and the antigenic clustering showed in this study mimics the phylogeny of the E protein. Second, neither serum samples and isolates of current WNV vaccines available for horses nor representative of other WNV lineages or other flaviviruses were used in the study. However, although the limited number of viral species, as far as we know this is the first large antigenic map assessed for flaviviruses belonging to the same serocomplex.

Results of the comparison between antigenic and amino acid sequences distances between viruses demonstrated that although there is considerable antigenic variation among viruses that are genetically similar, over 90% of the antigenic variation is predictable from the E protein amino acid sequence. Despite the clustering of the pairwise distances into three groups (caused by the three main antigenic subcomplexes) the estimate of the average number of amino acid substitutions causing a two-fold change in antibody titer is in line with previous studies on RNA viruses with single dominant surface glycoproteins, at 22 amino acids (Horton et al., 2010). The individual disproportionate effect of some amino acid substitutions at important epitopes or receptor binding sites was not captured here, yet these analyses demonstrate the opportunity of using this approach combined with reverse genetics to investigate those antigenically important sites. Overall, aa mutations observed between WNV L1 and L2 strains do not change the antigenicity of the E protein thus confirming the effectiveness of current WNV vaccines for horses.

Genetic characterization of circulating viruses is one of the key activities that most laboratories, even low-throughput, have in place. This scenario is further accentuated by the availability of next generation sequencing techniques (NGS). Indeed, the development of

innovative molecular technologies and of bioinformatics has greatly increased the production and release of sequence data and our knowledge of the evolution, transmission and pathogenicity of viruses. However, the analysis of antigenic differences within circulating viruses is not as timely as the genetic counterpart. In this perspective, we strongly believe that antigenic cartography needs to be coupled to NGS and genome characterization of circulating viruses. In this way, analysis of antigenic properties in a larger context (antigenic map) rather than one-by-one assays may help to predict the hazard related to the introduction of an "antigenically unknown" pathogen. Further antigenic analyses employing representative strains of extant serocomplexes are currently underway. This will provide a more in deep knowledge of the antigenic relationships between flaviviruses.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.11.023.

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