1 **Title:** Antigenic relationship among zoonotic flaviviruses from Italy

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22 **Abstract**

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

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49 INTRODUCTION

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

68 Viruses in the genus *Flavivirus* are placed into complexes with shared antigenic cross-reactivity.
69 WNV and USUV belong to the Japanese encephalitis virus (JEV) serocomplex along with viruses
70 including JEV, Murray Valley encephalitis virus and St. Louis encephalitis virus. Other serocomplexes
71 include yellow fever virus, DENV and tick-borne encephalitis virus, which contains three subtypes
72 and louping ill virus (Porterfield, 1980). This classification has largely been supported by genomic
73 phylogeny, but the correspondence between genetic and phenotypic expression is not clear. A
74 previous study demonstrated complex antigenic relationships among the flaviviruses, but used sera
75 from individuals that had been vaccinated against more than one virus, complicating interpretation
76 (Mansfield et al., 2011). Thus, flaviviruses constitute a challenge for serological diagnosis and
77 prediction of vaccine cross-protection. Indeed, specificity in flavivirus antibody-measurement is
78 significantly hampered by the structural similarity of the viruses and the resulting antibody cross-
79 reactivity. The antigenic characteristics of flaviviruses are mainly determined by their envelope (E)

protein structures (Heinz, 1986). This protein is widely used in serological flavivirus 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 et al., 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., 2011). Serological cross reaction has been demonstrated to occur also between WNV and USUV (Blazquez 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 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 show the antigenic relationships for a 33 strains of zoonotic flaviviruses (WNV and USUV) isolated in Italy in the last ten years, and compare with their genetic relationships.

MATERIALS AND METHODS

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 (Tab 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

110 in Italy were also included. Likewise, two USUV reference strains (939 and SAAR of the European
111 and African lineages, respectively) were also added. One JEV isolate was also used for the analysis.
112 Italian field strains used in this study represent all the isolates obtained between 2008 and 2015
113 within the framework activities of the National surveillance plan for WNV. Isolation was performed
114 following procedures previously described (Savini et al., 2013).

115 **Ethics**

116 All procedures on animals were accomplished respecting the European and Italian regulations on
117 the use of animals for experimental purposes. The study was approved by the Animal Welfare
118 Committee of IZSAM and authorized by the Italian Ministry of Health (authorization number
119 48/2015-PR).

120 **Inactivation and production of hyperimmune sera**

121 Low cell-passages (p4, onto African green monkey kidney cells-Vero) of the field strains were used
122 for inactivation. Inactivation was verified by propagating each virus onto Vero cells for three times
123 followed by immunofluorescence. Inactivated viruses for immunization were prepared at
124 approximately 10^6 tissue culture infective doses- TCID₅₀/ml per 20 ml with inactivation by binary
125 ethylenimine (BEI, 5mM; Ronchi et al., 2012) and addition of a commercial adjuvant (Montanide™
126 Gel, Seppic Srl, Milan-Italy) at a ratio of 1 : 1 (v/v) virus to adjuvant. Adult New Zealand white female
127 and male rabbits (of nearly 3.0 kg of weight) were employed for production of hyperimmune serum.
128 Rabbits were housed in wire cages (one individual per cage) under controlled environmental
129 conditions with free access to food and water. Prior immunization, blood was collected from all
130 animals in EDTA tubes and recovered plasma was screened to confirm the absence of WNV/USUV
131 antibodies by cELISA (ID Screen West Nile Competition Multi-species, IDvet- Grabels, France).
132 Rabbits were administered following sedation (Prequillan®, Fatro) and general anesthesia (Zoletil®,
133 Virbac® + Rompun®, Bayer) with the inactivated viruses. Two rabbits per virus were immunized at
134 day (d) 0 and d2 with inactivated virus combined with commercial adjuvant (1 ml in total) by
135 intramuscular (IM) and intraperitoneal (IP) routes. At d4, in addition to the IM administration,
136 inactivated virus and adjuvant (1 ml in total) were also given by a subcutaneous route. At d6,
137 inactivated virus and adjuvant (3 ml in total) were administered by IM with four different injections
138 of 0.75 ml each in the legs and shoulders. At d8, the same protocol of d6 was used. At d21,
139 serological screening by serum-neutralization (SN) with the homologous strain was adopted to

140 confirm the seroconversion of immunized animals. At the end of the vaccination period, rabbits
141 were humanely euthanized with Tanax® (Intervet).

142 **Serological assay**

143 Sera were heat inactivated at 56 °C. Serum samples from rabbits were tested by SN (Di Gennaro et
144 al., 2014) against all WNV, USUV and JEV isolates used for immunization of rabbits. Briefly, starting
145 from a dilution titer of 1:5, serial 2-fold dilutions were made in microtiter plates, and 100 tissue
146 culture infective doses (TCID) of antigen were added to each dilution. Thereafter, the mixtures were
147 incubated at 37°C for 1 h, and 10⁵ Vero cells were added to each well. The plates were incubated at
148 37°C for 5 days. Starting from the third day after incubation, the plates were checked for cytopathic
149 effect, and the antibody titer was defined as the reciprocal of the highest dilution of the serum that
150 showed 100% neutralization. The reciprocal of the ratio between heterologous and homologous SN
151 titers for individual serum samples was calculated to determine the fold difference between
152 heterologous and homologous reactions. SN plates were blinded to prevent bias.

153 **Characterization of the E protein**

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

159 **Phylogeny**

160 E protein sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy (MUSCLE
161 with default settings). The analysis was performed on the Phylogeny.fr platform
162 (<http://www.phylogeny.fr/index.cgi>). The phylogenetic tree was reconstructed using the maximum
163 likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The default substitution
164 model was selected assuming an estimated proportion of invariant sites (of 0.055) and 4 gamma-
165 distributed rate categories to account for rate heterogeneity across sites. The gamma shape
166 parameter was estimated directly from the data (gamma=1.649). Reliability for internal branch was
167 assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree
168 were performed with TreeDyn (v198.3).

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170 **Antigenic cartography**

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

185 **RESULTS**

186 **Serological assay**

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

195 **Antigenic relationships**

196 The strains of USUV, WNV and JEV used in this study were demonstrated to be in separate clusters
197 on the antigenic map (Figure 1). The total antigenic variation among the representatives of USUV

198 (three antigenic units, AU) was similar to the variation among the WNV isolates. Strains from WNV
199 L1 were, however, not antigenically distinguishable from L2. Although they belong to the same
200 serogroup, JEV was closer antigenically to the USUV (mean of 3.5 AU) than the WNV strains (mean
201 of 6 AU). The antigenic proximity of JEV with USUV concurred with the phylogeny of the E protein
202 (Figure 2). The apparent clustering of sera (grey cubes) together with colored dots (viruses) in the
203 3D map confirms that they were hyperimmune sera specifically produced for the selected viruses.
204 However, in few cases cross-reaction was revealed between WNV and USUV and between USUV
205 and JEV. Antigenic maps were consistent, and robust to addition/removal of viruses. Thus, antigenic
206 cartography provided, in this case, a reliable means of visualizing and quantifying the relationship
207 between the selected flaviviruses.

208 **Analysis of the E protein**

209 An overall aa identity of the E protein ranging from 99.4 to 100 % was demonstrated within WNV L1
210 strains, from 99.7 % to 100 % within WNV L2 strains, 93.3 % to 95.6 % between WNV L1 and L2
211 strains, 76.2 % to 78.0 % between WNV (regardless of the lineage) and USUV strains. Italian USUV
212 field strains were nearly identical within each other. By employing one representative isolate for
213 each viral genotype (Figure 3), aa relatedness with JEV E protein was 76 %, 77 % and 78 % for WNV
214 L1, L2 and USUV, respectively. However, aa identity in the D3 domain was higher (84 %) between
215 JEV and USUV with respect to that observed between homologous domains of JEV with WNV L1 (77
216 %) and WNV L2 (76 %). Aa identities between homologous D1 and D2 of USUV and JEV were 73 %
217 and 77 %, respectively.

218 **Predicting antigenic distance from amino acid sequence homology**

219 Antigenic distances measured from the antigenic map were compared with differences from an E-
220 protein amino acid similarity matrix. Each virus was compared with every other virus, giving an
221 estimate of the correlation between antigenic and amino acid distances. Pairwise comparison of all
222 antigenic distances with amino acid distances showed a statistically significant correlation ($r = 0.91$
223 (Figure 4). In addition, a linear regression model predicts on average, one unit of antigenic change,
224 equivalent to a 2-fold change in antibody titer, for every 22 amino acid substitutions in the E-protein
225 ectodomain.

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228 DISCUSSION

229 In this study, we investigated the antigenic relationship between a number of field WNV and USUV
230 strains which originated from biological samples collected in the last ten years during the activities
231 of a serological, entomological and virological surveillance program for West Nile neuroinvasive
232 disease implemented at the national level by the Italian Ministry of Health in order to control WNV
233 circulation. While isolates used for cartography were representative of both WNV L1 and L2, which
234 are the unique WNV lineages circulating in Italy and with distinct genomic signatures, USUV strains
235 were all nearly identical to each other as they belonged to the main European lineage. Reference
236 WNV and USUV strains and one JEV isolate were also included in the analysis. Whereas reference
237 WNV strains including Eg101, NY99 and B956 belong to L1 and L2, respectively, USUV strain 1776
238 isolated in the Republic of South Africa in 1959 is representative of the Africa 2 lineage (Cadar et al.,
239 2017).

240 The antigenic relationships were depicted in a 3D map. This map was created by interpreting serum-
241 neutralization results. The SN method was previously tested and compared to the official plaque
242 reduction neutralization test on human and equine serum samples by our group at IZSAM (Di
243 Gennaro et al., 2014). The map was demonstrated to be robust and consistent with phylogeny of
244 the E protein. Indeed, the map provided a reliable means of visualizing and quantifying the
245 relationship between these flaviviruses. JEV was demonstrated to be antigenically closer to USUV
246 than WNV as indicated also by phylogeny of the E protein. The highest % of aa sequence homology
247 between JEV and USUV strains was demonstrated in the D3 domain of the E protein. As this domain
248 has been indicated to be involved in cross-protection among flaviviruses, it is tempting to speculate
249 that the proximity of these viruses in the antigenic map is related to the observed homology in this
250 domain. Intriguingly, although cross reaction between JEV and WNV was not revealed in our
251 experimental settings, previous studies demonstrated cross reaction between JEV and WNV in vitro,
252 albeit with sera from individuals vaccinated against multiple agents (Mansfield et al., 2011) and in
253 vivo with a prominent role of D3 as inducer of protective antibody response in mice challenged with
254 a virulent WNV strain (Li et al., 2011). Nevertheless, antigenic cartography, which was previous
255 applied to other pathogens, allowed high resolution quantitative analyses and visualizations of
256 antigenic relationships between the tested field viruses. The viruses clearly grouped in three main
257 clusters and strains of WNV L1 were not antigenically distinguishable from WNV L2 strains. One
258 could reasonably point out that in a few cases, no cross-reactivity was evidenced between some

259 WNV strains. However, because antisera were tested against multiple antigens, and antigens tested
260 against multiple antisera, many measurements were used to determine the position of the antigen
261 and antiserum points in an antigenic map, thus increasing the accuracy of point placement beyond
262 that of individual SN measurements. One advantage of antigenic cartography is that it is minimally
263 dependent on individual variations in serological response compared with other interpretations of
264 antigenic data. Indeed, assays of the same strain and antiserum can occasionally produce different
265 values, as it is the case of this study, even in the same laboratory. Accordingly, although cross
266 reactions between WNV and USUV and between USUV and JEV were observed in a few cases, they
267 did not cluster together in the map.

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

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

289 Genetic characterization of circulating viruses is one of the key activities that most laboratories,
290 even low-throughput, have in place. This scenario is further accentuated by the availability of next
291 generation sequencing techniques (NGS). Indeed, the development of innovative molecular
292 technologies and of bioinformatics has greatly increased the production and release of sequence
293 data and our knowledge of the evolution, transmission and pathogenicity of viruses. However, the
294 analysis of antigenic differences within circulating viruses is not as timely as the genetic counterpart.
295 In this perspective, we strongly believe that antigenic cartography needs to be coupled to NGS and
296 genome characterization of circulating viruses. In this way, analysis of antigenic properties in a larger
297 context (antigenic map) rather than one-by-one assay may help to predict the hazard related to the
298 introduction of an “antigenically unknown” pathogen. Further antigenic analysis employing several
299 strains of representative serocomplex are currently underway. This will provide a more in deep
300 analysis of antigenic relationship between flaviviruses.

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305 is solely for the purpose of providing specific information and does not imply recommendation or
306 endorsement by the IZSAM.

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Figure Legend

Figure 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.

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

440 **Figure 3.** Alignment of the E proteins of one selected sequence per viral species. Red, D1 domain;
441 Blue, D2 domain; Green, D3 domain. Aa shared by USUV and JEV are highlighted in yellow.

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

448 **Supplementary material**

449 SN results which include homologous (yellow boxes) and heterologous reactions

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459 **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
19883	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	Insect	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		New York, USA	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				