



## Short communication

## West Nile virus in horses during the summer and autumn seasons of 2015 and 2016, Portugal



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## ARTICLE INFO

## Keywords:

West Nile virus  
WNV  
Horses  
Portugal

## ABSTRACT

West Nile fever (WNF) is an emergent disease in Europe, under surveillance in the European Union. Following a 5-year period of apparent silence (autumn 2010 to summer 2015), West Nile virus (WNV) reemerged in the South of Portugal, in July 2015.

Here we present data from the onset, geographic location within mainland Portugal, and outcome of clinical cases of WNV infection in horses in 2015 and 2016. During the transmission seasons of 2015 and 2016, twenty-seven horses, most symptomatic ( $n = 20$ ) were found positive to IgM, pr-E immunoglobulins and VNT, leading to the subsequent report to Animal Disease Notification System of the European Commission (ADNS) by the Portuguese National Authority for Animal Health.

Outbreaks occurred in the middle summer (August) and early/mid autumn (October/November) of 2015 and 2016, in the southern regions of the country (Alentejo and Algarve).

Compared with the previous WNV transmission seasons of 2004 and 2010, a higher number of cases were reported in 2015 and 2016.

The results of our study contribute to increase information concerning the geographic areas affected and time period for WNV transmission risk in Portugal.

## 1. Introduction

West Nile virus (WNV) is an arthropod-borne, single-stranded positive-sense RNA virus that belongs to the Japanese encephalitis virus (JEV) serocomplex within the *Flaviviridae* family. WNV transmission cycle includes a wide range of bird species as natural reservoirs, mosquitoes as biological vectors, and humans and equines as dead-end hosts. Horses can become infected when bitten by a mosquito-carrying virus, but they do not contribute to the spread or amplification of WNV in natural cycle, as the low level viremia is insufficient to contribute to the amplification cycle (Bunning et al., 2002). Furthermore, this short viraemic phase (around 4–6 days) occurs during the incubation period which may vary from 3 to 15 days. Seroconversion occurs 5 to 7 days post-infection (Bunning et al., 2002). Most horses seroconvert without clinical disease. Only around 10% of the infected horses show clinical symptoms (Ostlund et al., 2000). The first symptoms are mostly unspecific and include fever, depression, loss of appetite and colic. When

infection proceeds symptoms usually include encephalitis with ataxia as well as limb weakness, recumbency and muscle fasciculation (Ostlund et al., 2000).

Acute WNV infections in equines can be diagnosed by the detection of the virus genome by RT-PCR or presence of IgM specific antibodies by ELISA. However, the detection of viral RNA by RT-PCR is difficult, due to the virus' low-level, short term viraemia in horses. Therefore, diagnosis of WNV in horses is commonly achieved by serological tests, demonstrating the presence of IgM antibodies.

Since 2010, WNV infections among horses were repeatedly reported in the Mediterranean basin (OIE disease information). In Portugal, in the summer of 2004, two linked WNV cases were reported in Irish tourists that had participated in a bird watching tour in Algarve (Connell et al., 2004). After these human cases, the Portuguese National Authority for Animal Health (Direção-Geral de Alimentação e Veterinária- DGAV) set up a regional surveillance program, implemented in risk areas (wetlands and bird sanctuaries located in Algarve region),

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**Table 1**

Horse serum samples from all over the country received at the laboratory (INIAV) for diagnosis of WNV in the years 2015 and 2016 (Clinical suspicions of WNF and cohabitants).

| Serological Results           |                    |                    |                 |               |               |              |             |  |               |                               |
|-------------------------------|--------------------|--------------------|-----------------|---------------|---------------|--------------|-------------|--|---------------|-------------------------------|
| Year 2015                     |                    |                    |                 |               |               |              |             |  |               |                               |
| Location NUTS II <sup>a</sup> | Municipality       | Date of collection | N°. of Premises | IgMs          | prE-IgGs      | VNT          | Titre range | Neurol. signs/<br>Confirmed Clinical cases | Case fatality | Outbreaks (ADNS) <sup>b</sup> |
|                               |                    |                    |                 | Pos/Tested    | Pos/Tested    | Pos/Tested   |             |  |               |                               |
| Algarve                       | Loulé <sup>c</sup> | Aug–Nov            | 13              | 7/45          | 23/45         | 22/23        | > 10–640    | 5/2  |               | 2 (31 Aug 2015)               |
|                               | Faro               | Aug–Oct            | 3               | 1/10          | 1/10          | 1/1          | 40          | 3/1  |               | 1 (31 Aug–2015)               |
|                               | Olhão              | Sept               | 1               | 1/15          | 1/15          | 1/1          | 160         | 2/1  |               | 1 (28-Sept-2015)              |
|                               | Albufeira          | Sept               | 1               | 0/1           | 0/1           | –            | –           | 0  |               | –                             |
|                               | Lagos              | Sept–Oct           | 3               | 3/19          | 9/19          | 8/9          | > 10–160    | 2/1  | 1             | 1 (18-Sept-2015)              |
| Alentejo                      | Silves             | Oct                | 1               | 0/2           | 0/2           | –            | –           | 1/0  |               | –                             |
|                               | S. do Cacém        | Sept–Oct           | 2               | 0/7           | 0/7           | –            | –           | 2/0  |               | –                             |
|                               | Á. do Sal          | Sept–Oct           | 4               | 3/8           | 4/8           | 4/4          | 40–160      | 6/3  | 1             | 2 (24-Sept-2015 14-Oct-2015)  |
|                               | Arronches          | Oct                | 1               | 1/1           | 1/1           | 1/1          | 160         | 1/1  |               | 1(15–10-2015)                 |
|                               | Alpiarça           | Oct                | 1               | 1/1           | 1/1           | 1/1          | 320         | 1/1  |               | –                             |
| A.M.Lisboa                    | Évora              | Oct                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               | –                             |
|                               | Benavente          | Nov                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               | –                             |
|                               | Portalegre         | Nov                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               | –                             |
|                               | Seixal             | Dez                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               | –                             |
|                               | <b>Total</b>       |                    | <b>34</b>       | <b>17/113</b> | <b>40/113</b> | <b>38/40</b> |             | <b>27/10</b>                               | <b>2</b>      | <b>8</b>                      |
| Year 2016                     |                    |                    |                 |               |               |              |             |  |               |                               |
| Algarve                       | Loulé              | Fev–Oct            | 3               | 1/5           | 2/5           | 2/2          | 20          | 4/1  | 1             | 1 (30-Aug-2016)               |
|                               | Lagos              | July–Dec           | 2               | 1/2           | 1/2           | 1/1          | 160         | 2/1  | 1             | 1 (14-Nov-2016)               |
|                               | Silves             | Oct–Dec            | 3               | 2/3           | 3/3           | 3/3          | 80          | 3/2  | 1             | 2 (13-Oct 2016 18-Oct-2016)   |
| Alentejo                      | Odemira            | May                | 1               | 0/5           | 1/5           | 1/1          | 80          | 5/0  |               |                               |
|                               | S. de Magos        | Jun–Sept           | 2               | 0/2           | 1/2           | 1/1          | 320         | 2/0  |               |                               |
|                               | Portalegre         | Sept–Nov           | 2               | 2/4           | 2/4           | 2/2          | 20          | 4/2  |               |                               |
|                               | Álter-do-Chão      | Oct                | 1               | 1/1           | 1/1           | 1/1          | 40          | 1/1  | 1             |                               |
|                               | Benavente          | Nov                | 1               | 1/1           | 1/1           | 1/1          | 160         | 1/1  | 1             |                               |
|                               | Elvas              | Nov–Dec            | 1               | 1/2           | 1/2           | 1/1          | 80          | 2/1  |               | 1(8-Nov-2016)                 |
|                               | Évora              | Nov                | 2               | 0/2           | 0/2           | –            | –           | 2/0  |               |                               |
|                               | Beja               | Nov–Dec            | 2               | 1/3           | 1/3           | 1/1          | 40          | 1/1  |               |                               |
| A.M.Lisboa                    | Seixal             | Apr                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               |                               |
|                               | Moita              | Dec                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               |                               |
| Centro                        | Alcanena           | Apr                | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               |                               |
| Norte                         | Fafe               | Sept               | 1               | 0/1           | 0/1           | –            | –           | 1/0  |               |                               |
| <b>Total</b>                  |                    |                    | <b>24</b>       | <b>10/34</b>  | <b>14/34</b>  | <b>14/14</b> |             | <b>33/10</b>                               | <b>5</b>      | <b>5</b>                      |

<sup>a</sup> NUTS – Nomenclature of territorial units for statistics (Eurostat-GISCO 2014).<sup>b</sup> ADNS- Animal Disease Notification System of the European Commission (ADNS). Notifications sent by DGAV.<sup>c</sup> Municipality where the human case was registered. Pos- positive samples.

including testing horse sera, and checking for increased mortality in susceptible bird species. Through this program, neutralizing antibodies were detected in nine horses and two birds, although neither symptomatic horses nor bird mortality were found (Barros et al., 2011). From 2005 to middle 2010, no human or horse clinical cases were reported in the country. In 2010, another WNV human case was identified (Alves et al., 2012) and horse clinical cases were also reported (Barros et al., 2011).

After a five years period of epidemiological silence, in the summer of 2015, a case of West Nile Neuroinvasive Disease (WNND) was diagnosed in a man living in the Algarve region (Ze-Ze et al., 2015).

Here we presented data from the onset, geographic location within mainland Portugal, and outcome of clinical cases of WNV infection in horses in 2015 and 2016. Also, data from the laboratory serosurvey, conducted at the National Institute for Agrarian and Veterinary Research (INIAV, I.P.) between 2011 and April 2015, comprising the serological testing of 989 horse serum originated all over the country is divulged.

## 2. Materials and methods

### 2.1. Case definition

For the purposes of this study, a horse case was defined according with OIE by (1) the presence of one or more of signs of WNV clinical disease (ataxia, recumbency, paresis, paralysis or death) and (2) positive to IgM ELISA or RNA WNV by RT-PCR. Once a clinical case was identified, the regional services of the veterinarian authorities (DGAV) visited the holding to collect samples and to conduct an epidemiological survey concerning the animal's travel history, the exact location and the vaccination status. Information on the cohabitants was also gathered. In addition, any occurrence of death birds on the surroundings was investigated.

### 2.2. Serological tests

Horse sera were tested for WNV antibodies. IgMs and total antibodies against the viral envelope protein E (pr-E), mainly IgGs (therefore hereafter referred to as pr-E IgGs), but also other immunoglobulin classes were investigated with two commercial competition enzyme linked immunosorbent assays (ELISAs), following the manufacture

instructions. The ID screen West Nile IgM Capture (IDvet, France) and ID Screen West Nile competition (IDvet, France) kits were used, respectively, for IgM and pr-E IgGs detection. Due to cross-reactivity with other Flaviviruses, samples that tested positive in the pr-E ELISAs were subsequently investigated by virus microneutralization test (VNT). Vero E6 cells and West Nile virus Egyptian Eg-101 topotype strain (Melnick et al., 1951), a representative of WNV lineage 1 (clade 1a), were used in the VNT, performed according to the OIE standard procedure (OIE Terrestrial manual, West Nile Fever. Chapter 2.1.24, 2013).

Samples are considered positive for WNV IgG antibodies when testing positive in the pr-E ELISAs and showing an antibody titers  $\geq 1:10$  in the VNT. Samples that test positive to pr-E IgGs and negative in VNT may contain antibodies against other viruses of the JEV serocomplex, and were considered negative.

### 2.3. Virological tests

For WNV-RNA detection, a specific RT-qPCR targeting the NS2A genomic region was used (Barros et al., 2013). Viral isolation was attempted in Vero E6 cells cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

## 3. Results

### 3.1. West Nile fever transmission season in Portugal 2015 and 2016

During the summer and autumn seasons of 2015 and 2016, a total of 147 horse sera (113 from 2015 and 34 from 2016) were received for laboratorial screening of WNV. Of these 147 samples, 60 originated from clinically suspected animals and the remaining from cohabitants. Most samples originated from the Algarve and Alentejo regions (Table 1).

In the year 2015, WNV pr-E IgGs antibodies were found in 40 animals (35.4%), 38 also confirmed positive by VNT (Table 1). Of the 38 specific pr-E IgG positive samples, 17 were also WNV IgM positive. In total, 15% of the animals tested had WNV specific IgM antibodies. Among these animals, 10 showed neurological clinical signs and were considered clinical cases (Table 1), while 7 were found asymptomatic. Of the 10 symptomatic horses, 8 recovered, one died and one was euthanized for welfare reasons (20% fatality rate).

The date of clinical signs onset for the first WNV IgMs-positive clinical case was August 24, 2015. The last recorded onset of clinical signs was October 10, 2015. As the season progressed to winter no more cases were reported. All clinical cases were reported in the southern region of the country, south of the Tagus River (Fig. 1).

During the year 2016, 34 horse sera were received for laboratory diagnosis of WNV. Antibodies against the WNV pr-E IgGs were found in 14 animals (41% positivity), all of them confirmed by VNT. From these, 10 animals also tested positive for IgMs, revealing that almost 30% of these horses had been recently infected (Table 1). As in 2015, all IgM positive animals were also positive for pr-E IgGs antibodies.

The date of onset of the clinical signs for the first WNV IgMs-positive clinical case was August 25, 2016. The last recorded date of onset of clinical signs was November 18, 2016. As in 2015, the first clinical case was registered by the end of August, in the same geographic area (Municipality of Loulé, Algarve). However, while in 2015 the last reported clinical case was registered in mid-October, in 2016 the mosquito transmission season was extended until mid-November. The last season cases were reported in animals from Alentejo region near the Spanish border (Arronches and Elvas, in 2015 and 2016, respectively) (Fig. 1), suggesting that meteorological conditions in these regions during mid-autumn were suitable for mosquito-borne transmission of WNV.

All the 10 WNV-IgM positive horses screened in 2016 showed neurological signs consistent with WNV infection, and were considered clinical cases (Table 1). From those animals, three died, two were

sacrificed due of irreversible complications (50% case-fatality rate) and five recovered completely.

Clinical signs such as high pyrexia, paralysis, anorexia, ataxia, depression, hyperesthesia of hindquarters, recumbency, and muscle fasciculation were frequently observed in IgM-positive animals.

Of the 20 clinical cases, observed in 2015 and 2016, nine were females and 11 males. Although not statistically significant ( $p$ -value = 0.083), the case fatality rate was higher in females (5/9; 55.5%) than in males (2/11; 18.1%).

WNV-RNA was not detected in any of the 147 samples tested by real time RT-PCR, and all attempts for virus isolation in Vero E6 cells failed. With the exception of one case, the search for viral RNA in biological matrices other than blood, in the animals that died was not possible since owners did not allow the collection of tissue samples, hampering further analysis.

The epidemiological enquire conducted by DGAV showed that none of the confirmed clinical cases had traveled outside the country, demonstrating that infection took place in the national territory.

Unlike 2015, no human cases were reported in the country in the year 2016.

### 3.2. WNV surveillance (2011–2016)

In the laboratory serosurvey carried out between 2011 and 2016, a total of 989 horse sera samples were screened for WNV antibodies. These samples originated from animals that had no clinical suspicion of WNV and arrived to the laboratory for serological diagnosis of viral infections other than WNV. Pr-E IgGs were detected in 44 of the 989 horses tested (4.4%), of which only 26 (1.8%) were confirmed by VNT, showing antibody titers ranging from 32 to 640 (Table 2). WNV-IgMs were not detected in any horse sample tested. A few horses ( $n = 24$ ) were analysed by RT-qPCR, all testing negative (Table 2). No clinical cases were reported in the country between 2011 and 2014.

## 4. Discussion

Following a 5-year period of apparent silence (between 2010 and early 2015), West Nile virus reemerged in Portugal in the summer 2015, in the Algarve region, a previously recognized risk zone for WNV circulation (Barros et al., 2011; Esteves et al., 2005).

Following a human case report, DGAV, promoted sensitization campaigns at the regional level to avoid under diagnosis, by increasing awareness of WNV infection among veterinarians and horse owners. In response to these alerting measures, samples from WNV horse suspicions as well as from horses sharing the same premises were collected and sent to INIAV, between August and October 2015 (summer and autumn months), for analysis. The seasonality, distribution, and prevalence of vector-borne diseases are significantly influenced by climate factors, primarily high and low temperature extremes and precipitation patterns. During the winter/spring months the meteorological conditions are usually unsuitable for vector activity.

By late-August 2016, a new outbreak in horses was declared in the same Algarve municipality as in the previous year (Loulé). In total, as in 2015, 10 clinical cases were identified in the country, from Algarve and Alentejo. However, the case fatality rate was higher in 2016 (50%) than in 2015 (20%). More females than males were found affected, in accordance to reports from other authors who described females being 2.9 times more likely to succumb to infection than males (Salazar et al., 2004).

All WNV human and horse cases described so far in Portugal occurred in the southern region of the country where climatic and environmental factors favor WNV dissemination. The Alcácer-do-Sal municipality, crossed by the Sado River, comprehends large areas of rice paddies of wetland that provide rich habitats for mosquitoes. In the Algarve, several lagoons integrated in the wetland of the coast provide habitats for many species of mosquitoes (Freitas et al., 2012). These two

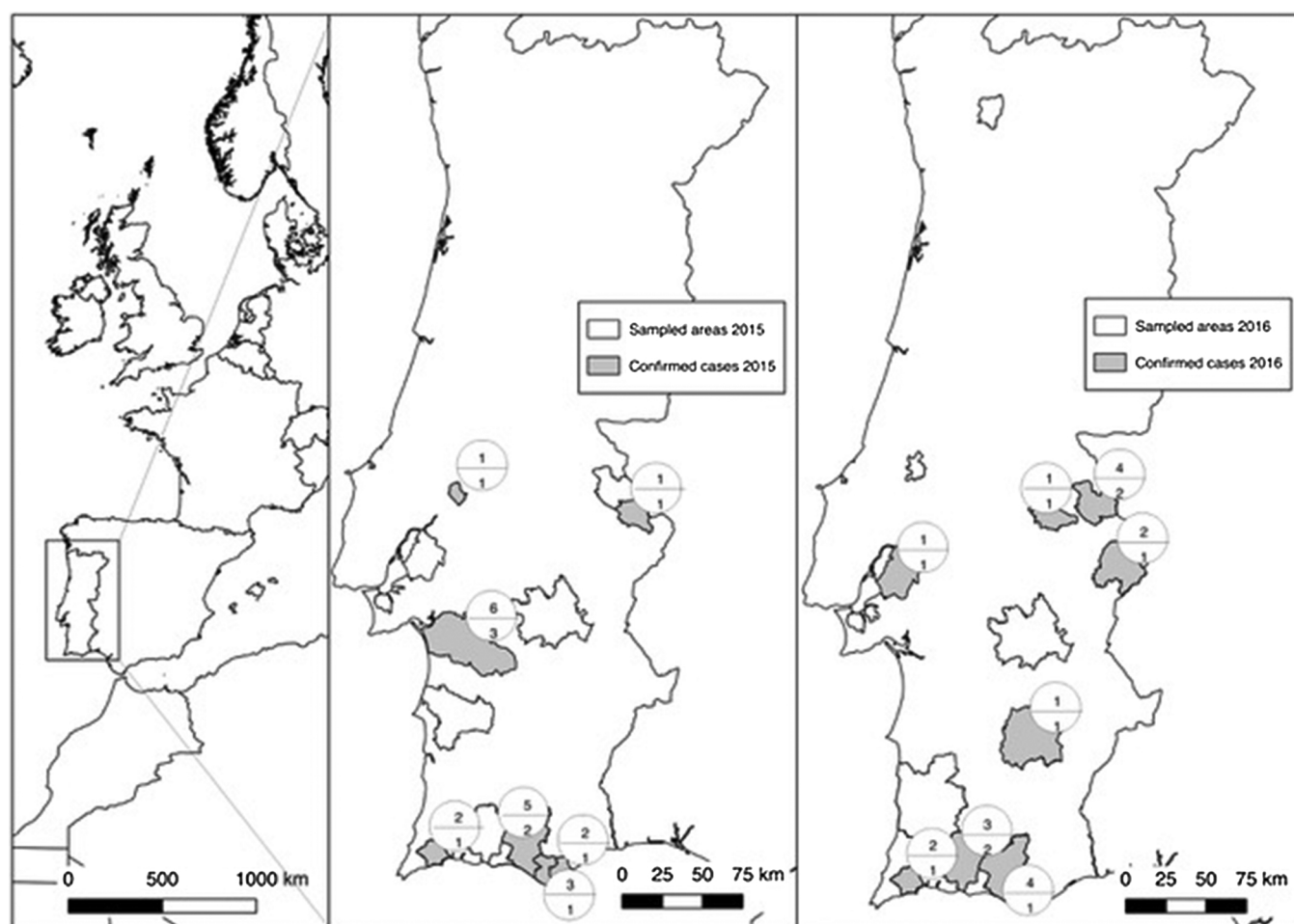


Fig. 1. Sampled areas and areas with confirmed cases of WNV in 2015 and 2016. Number of horses with neurological signs (upper half of circles) vs. number of confirmed cases (lower half of circles). © EuroGeographics for the administrative boundaries. The geographical distribution map was constructed using Quantum Geographic Information System (QGIS) software 2.0.1.

**Table 2**

Laboratory active surveillance carried out between 2011 and 2016 in serum samples of horses from all over the country that arrived to the laboratory for serological diagnosis of viral infections other than WNV.

| Year of collection | pr-E IgGs     | IgMs        | VNT <sup>a</sup> | Titer range | RT-qPCR     |
|--------------------|---------------|-------------|------------------|-------------|-------------|
|                    | P/T           | P/T         | P/T              |             | P/T         |
| 2011               | 11/201        | 0/11        | 4/11             | 80–128      | 0/8         |
| 2012               | 4/239         | 0/4         | 2/4              | 32–640      | 0/5         |
| 2013               | 9/162         | 0/9         | 7/9              | 32–256      | 0/3         |
| 2014               | 0/145         | –           | –                | –           | 0/4         |
| 2015               | 3/119         | 0/3         | 1/3              | 128         | –           |
| 2016               | 17/123        | 0/17        | 4/17             | > 10–640    | 0/4         |
| <b>Total</b>       | <b>44/989</b> | <b>0/44</b> | <b>18/44</b>     | <b>–</b>    | <b>0/24</b> |

P-positive samples; T-Total number of samples tested.

<sup>a</sup> VNT performed with a lineage 1 virus (Egypt Eg-101).

geographic regions also host large populations of resident and migratory aquatic birds. In Portugal, as in other countries with a temperate climate, mosquitoes' activity and density peaks in the summer and extends until autumn (Alves et al., 2010).

From horses that showed clinical signs, a case-fatality rate of 35% was observed, in agreement with other reports, which described that percentage around 25% to 50% of animals that show clinical signs die or require euthanasia (Murgue et al., 2001; Ostlund et al., 2000).

The fact that all samples were collected after the onset of the neurological signs may have accounted for the unsuccessful detection of

WNV-RNA and for the failure in the attempts of isolating the virus in cell cultures. At this stage of the infection, the low ( $10^3$  pfu/ml) and short (up to six days) viremia developed in horses, demonstrated in experimental infections (Bunning et al., 2002), poses significant obstacles to viral detection based diagnosis.

The lack of the molecular characterization of the strain causing the outbreaks constituted the major containment of this study. Traditionally, WNV lineage 1 strains were responsible for the majority of the outbreaks in Europe (Di Sabatino et al., 2014). However, in recent years, WNV lineage 2 has been spreading and causing disease outbreaks in humans and animals (Bakonyi et al., 2006; Bagnarelli et al., 2011; Danis et al., 2011; Sirbu et al., 2011).

The twenty-eight horses that tested pr-E IgG positive and VNT-negative may represent the contact with other JEV serocomplex viruses (Beck et al., 2017). Also, since sera having a virus neutralization titre of < 10 were considered negative, weakly positive sera in ELISA could become negative in VNT. As described by others, VNT offers higher specificity but appears less sensitive than ELISAs, for WNV diagnosis (Beck et al., 2017).

Between 2011 and 2016 the laboratorial surveillance showed that only 1.8% of the horses tested positive for both pr-E IgGs and VNT but negative for IgM. The presence of IgM antibodies is the best serological indication for a recent infection, as in horses they were shown to persist less than three months (Castillo-Olivares and Wood, 2004). Since WNV-IgGs are known to persist for long periods (Murgue et al., 2001) and no information on the vaccination and travelling history was available for these pr-E IgGs positive horses, a conclusion on the time and place of



infection, or putative vaccination, was hindered.

Since 2005, when the first outbreak was confirmed in the country, no abnormal bird death was observed.

In the rest of the Iberia, WNV activity has been reported in southern Spain. The first human clinical case of WNV infection in Spain was reported in 2004 in a patient visiting Southwestern Spain (Kaptoul et al., 2007). In 2007, WNV-lineage 1 was isolated in free-living and captive Spanish golden eagles in south-central Spain (Jimenez-Clavero et al., 2007). In 2010, two new cases of human WNF were confirmed in Andalucía (south of Spain) (García-Bocanegra et al., 2011) and in the same area where outbreaks in horses have been reported to OIE since 2010–2016.

In Portugal, RT-PCR evaluations of mosquitoes were negative to WNV-RT-PCR (Almeida et al., 2010; Alves et al., 2010).

## 5. Conclusion

This study confirms the circulation of WNV, during 2015–2016, in Portugal. The results contribute to increase information concerning the geographic areas affected by the virus.

Ongoing combined surveillance in birds, mosquitoes and horses is essential for the rapid diagnosis, for risk factor evaluation, as well as to support the implementation of control measures aiming the early identification of WNV circulation, before the onset of human disease.

## Acknowledgments

We thank the Regional Veterinary Authorities of Alentejo, in particular to Dr Maria Luisa Alegre and the private veterinarians Dr Miguel Bliebernicht, Dr Rui Martelo, Dr Elisa Bettencourt, Dr Constança Sepúlveda and Dr Pedro Caetano for their prompt and generous collaboration.

## References

- Almeida, A., Freitas, F., Novo, M., Sousa, C., Rodrigues, J., Alves, R., Esteves, A., 2010. Mosquito surveys and west nile virus screening in two different areas of southern Portugal, 2004–2007. *Vector-Borne Zoonotic Dis.* 10, 673–680.
- Alves, M.J., Osório, H., Ze-Ze, L., Amaro, F., 2010. Relatório Revive 2008/2009. Programa Nacional de Vigilância de Vetores Culicídeos. DDI, INSA, INSA, Lisboa.
- Alves, M.J., Poças, J.M., Luz, T., Amaro, F., Zé-Zé, L., Osório, H.C., 2012. West Nile virus infection in Portugal: considerations about a clinical case with febrile syndrome and rash. *Rev. Port Doenças Infec.* 8, 46–51.
- Bagnarelli, P., Marinelli, K., Trotta, D., Monachetti, A., Tavio, M., Del Gobbo, R., Capobianchi, M., Menzo, S., Nicoletti, L., Magurano, F., Varaldo, P., 2011. Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011. *Euro Surveill.* 16 (43) (pii: 20002).
- Bakonyi, T., Ivanics, E., Erdelyi, K., Ursu, K., Ferenczi, E., Weissenböck, H., Nowotny, N., 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg. Infect. Dis.* 12, 618–623.
- Barros, S.C., Ramos, F., Fagulha, T., Duarte, M., Henriques, M., Luis, T., Fevereiro, M., 2011. Serological evidence of West Nile virus circulation in Portugal. *Vet. Microbiol.* 152, 407–410.
- Barros, S.C., Ramos, F., Ze-Ze, L., Alves, M.J., Fagulha, T., Duarte, M., Henriques, M., Luis, T., Fevereiro, M., 2013. Simultaneous detection of West Nile and Japanese encephalitis virus RNA by duplex TaqMan RT-PCR. *J. Virol. Methods* 193, 554–557.
- Beck, C., Lowenski, S., Durand, B., Bahuon, C., Zientara, S., Lecollinet, S., 2017. Improved reliability of serological tools for the diagnosis of West Nile fever in horses within Europe. *PLoS Negl. Trop. Dis.* 11 (9), e0005936.
- Bunning, M.L., Bowen, R.A., Cropp, C.B., Sullivan, K.G., Davis, B.S., Komar, N., Godsey, M.S., Baker, D., Hettler, D.L., Holmes, D.A., Biggerstaff, B.J., Mitchell, C.J., 2002. Experimental infection of horses with West Nile virus. *Emerg. Infect. Dis.* 8, 380–386.
- Castillo-Olivares, J., Wood, J., 2004. West Nile virus infection of horses. *Vet. Res.* 35, 467–483.
- Connell, J., McKeown, P., Garvey, P., Cotter, S., Conway, A., O'Flanagan, D., O'Herlihy, B.P., Morgan, D., Nicoll, A., Lloyd, G., 2004. Two linked cases of West Nile virus (WNV) acquired by Irish tourists in the Algarve, Portugal. *EuroSurveillance* 8, 2517.
- Danis, K., Papa, A., Theodoropoulos, G., Douglas, G., Athanasiou, M., Detsis, M., Baka, A., Lytras, T., Mellou, K., Bonovas, S., Panagiotopoulos, T., 2011. Outbreak of West Nile virus infection in Greece, 2010. *Emerg. Infect. Dis.* 17, 1868–1872.
- Di Sabatino, D., Bruno, R., Sauro, F., Danzetta, M.L., Cito, F., Iannetti, S., Narcisi, V., De Massis, F., Calistri, P., 2014. Epidemiology of West Nile disease in Europe and in the Mediterranean Basin from 2009 to 2013. *BioMed Res. Int.*
- Esteves, A., Almeida, A.P., Galao, R.P., Parreira, R., Piedade, J., Rodrigues, J.C., Sousa, C.A., Novo, M.T., 2005. West Nile virus in Southern Portugal, 2004. *Vector Borne Zoonotic Dis.* 5, 410–413.
- Freitas, F.B., Novo, M.T., Esteves, A., de Almeida, A.P., 2012. Species composition and WNV screening of mosquitoes from lagoons in a wetland area of the algarve, Portugal. *Front. Physiol.* 2, 122.
- García-Bocanegra, I., Jaén-Téllez, J.A., Napp, S., Arenas-Montes, A., Fernández-Morente, M., Fernández-Molera, V., Arenas, A., 2011. West Nile fever outbreak in horses and humans, Spain, 2010. *Emerg. Infect. Dis.* 17, 2397–2399.
- Jimenez-Clavero, M.A., Tejedor, C.G., Rojo, G., Soriguer, R., Figuerola, J., 2007. Serosurvey of West Nile virus in equids and bovids in Spain. *Vet. Rec.* 161, 212.
- Kaptoul, D., Viladrich, P.F., Domingo, C., Niubo, J., Martínez-Yelamos, S., De Ory, F., Tenorio, A., 2007. West Nile virus in Spain: report of the first diagnosed case (in Spain) in a human with aseptic meningitis. *Scand. J. Infect. Dis.* 39, 70–71.
- Melnick, J.L., Paul, J.R., Riordan, J.T., Barnett, V.H., Goldblum, N., Zabin, E., 1951. Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. *Proc. Soc. Exp. Biol. Med.* 77, 661–665.
- Murgue, B., Murri, S., Zientara, S., Durand, B., Durand, J.P., Zeller, H., 2001. West Nile outbreak in horses in southern France, 2000: the return after 35 years. *Emerg. Infect. Dis.* 7, 692–696.
- OIE, 2013. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017*. [http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/2.01.24.WEST\\_NILE.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.24.WEST_NILE.pdf).
- Ostlund, E.N., Andresen, J.E., Andresen, M., 2000. West Nile encephalitis. *Vet. Clin. North Am. Equine Pract.* 16, 427–441.
- Salazar, P., Traub-Dargatz, J.L., Morley, P.S., Wilmot, D.D., Steffen, D.J., Cunningham, W.E., Salman, M.D., 2004. Outcome of equids with clinical signs of West Nile virus infection and factors associated with death. *J. Am. Vet. Med. Assoc.* 225, 267–274.
- Sirbu, A., Ceianu, C.S., Panculescu-Gatej, R.I., Vazquez, A., Tenorio, A., Rebreanu, R., Niedrig, M., Nicolescu, G., Pistol, A., 2011. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *Euro Surveill.* 16.
- Ze-Ze, L., Proenca, P., Osorio, H.C., Gomes, S., Luz, T., Parreira, P., Fevereiro, M., Alves, M.J., 2015. Human case of West Nile neuroinvasive disease in Portugal, summer 2015. *Euro Surveill.* 20.