

## SHORT REPORT

# West Nile virus lineage 2 in Sardinian wild birds in 2012: a further threat to public health

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Received 3 October 2012; Final revision 12 December 2012; Accepted 21 December 2012;  
first published online 24 January 2013

## SUMMARY

West Nile virus (WNV) strains belonging to lineage 2 were detected and isolated from the tissues of a goshawk and two carrion crows in Sardinia in August 2012. According to NS3 sequence analysis, the Sardinian isolates shared a high level of similarity with those of Italian lineage 2 strains which circulated in 2011 and with the homologous sequence of the 2004 Hungarian isolate. Following the human fatality reported in 2011 in Olbia, this study is the first to report the spread and enzootic circulation of WNV lineage 2 in Sardinia.

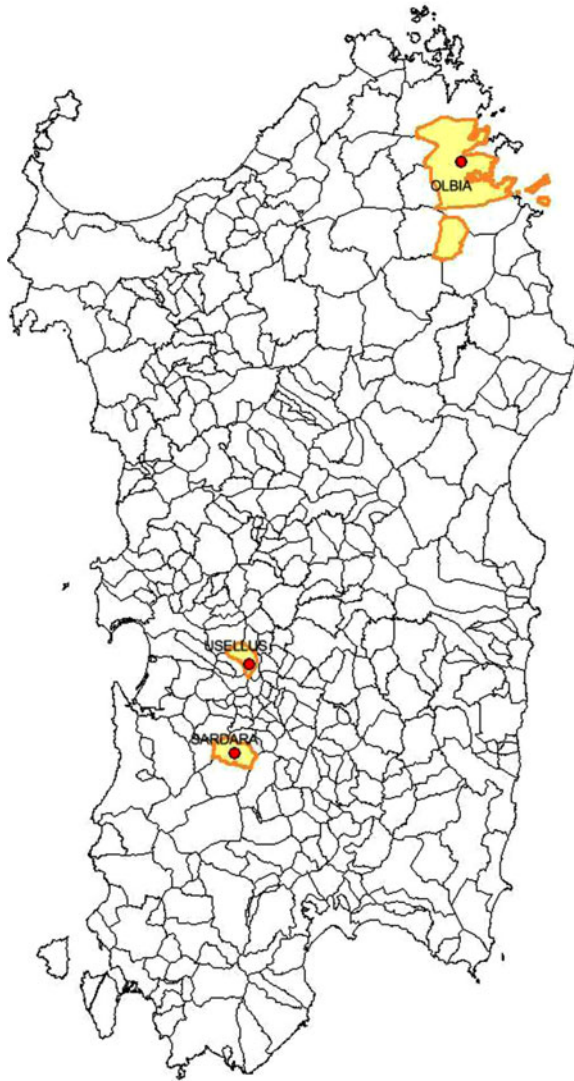
**Key words:** Veterinary epidemiology, veterinary virology, virus infection, West Nile virus, zoonoses.

West Nile virus (WNV) first appeared in Italy in 1998. Although the infection caused severe disease and deaths in horses, no cases were reported in humans at that time [1]. However, this scenario changed markedly with an increasing occurrence of neurological diseases due to WNV infection in both humans and horses [2]. In order to monitor and control WNV circulation, a serological, entomological and virological surveillance programme for West Nile neuro-invasive disease (WNND) was implemented at the national level by the Italian Ministry of Health [3, 4]. Within this framework WNV lineage 2 was detected in three wild birds in Sardinia. This report is the first to describe the circulation of WNV lineage 2 in the

wild bird population of Sardinia, providing evidence of the overwintering, spread and establishment of an enzootic cycle in the area of the WNV strain which probably caused the death of a human patient in 2011 in Olbia [2].

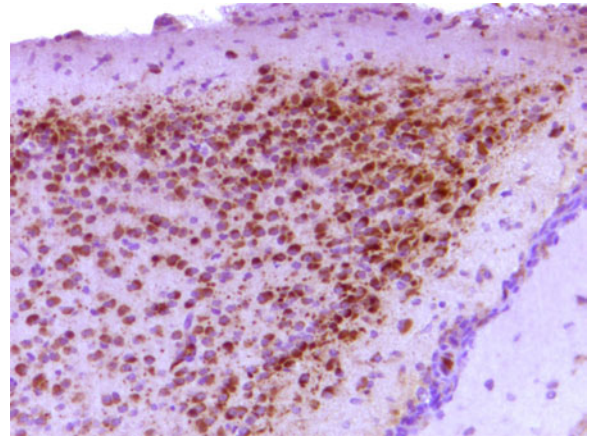
WNV RNA was detected in the tissues of two carrion crows (*Corvus corone corone*) and a northern goshawk (*Accipiter gentilis*) collected in three different municipalities of the Sardinian Region within the framework of the 2012 WNV surveillance activities (Fig. 1). The first carrion crow was caught on 6 August in Olbia (OT) (40955959 N, 9483152 E) while the second was caught on 8 August in the area surrounding Sardara (VS) (39370 N, 8500 E). Both carrion crows were caught during the culling activities performed to reduce the bird population in that area. The birds did not show any clinical signs. The northern goshawk was rescued in the vicinity of Usellus (OR) on 10 August (39483852 N, 851612 E). This bird

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**Fig. 1.** Map illustrating the geographical origin of the three birds infected with WNV lineage 2. (Source: Osservatorio Epidemiologico Veterinario Nazionale.)

exhibited severe neurological signs and died a few hours later. A complete necropsy was performed on the three birds and samples of brain, kidney, spleen and heart were taken, and homogenized in phosphate-buffered saline with antibiotics. From these samples, RNA was extracted and amplified by a WNV-specific real-time RT-PCR able to detect lineages 1 and 2, but not able to differentiate between these lineages [5]. The lineage was later identified by using lineage-specific RT-PCRs [6, 7]. Tissue homogenates were inoculated into confluent monolayers of C6/36 (*Aedes albopictus*) and VERO cell lines [8]. Fifty microlitres of brain homogenate of the goshawk were also intracerebrally inoculated into 16 suckling CD1 mice. Formalin-fixed and paraffin-embedded sections of the



**Fig. 2.** Immunohistochemical staining of a brain section of a suckling mouse infected with lineage 2 West Nile virus (WNV) strain. Large amounts of WNV antigen are seen in the cytoplasm of neurons residing within the cerebral cortex. Immunohistochemistry with Mayer's haematoxylin counterstain. Final magnification,  $\times 200$ .

Hungary 2004	TRVVAEMSEALRGLPIRYQTSAVHREHSGNEIVDMCHATLTH
Greece 2010	TRVVAEMSEALRGLPIRYQTSAVP <sup>RE</sup> HSGNEIVDMCHATLTH
ccOlbia/IT2012	TRVVAEMSEALRGLPIRY <sup>HT</sup> SAVHREHSGNEIVDMCHATLTH
ccSardara/IT2012	TRVVAEMSEALRGLPIRY <sup>HT</sup> SAVHREHSGNEIVDMCHATLTH
ngUssellus/IT2012	TRVVAEMSEALRGLPIRYQTSAVHREHSGNEIVDMCHATLTH
Ancona 2011	TRVVAEMSEALRGLPIRYQTSAVHREHSGNEIVDMCHATLTH
Italy, North East 2011	TRVVAEMSEALRGLPIRYQTSAVHREHSGNEIVDMCHATLTH

▲
▲

**Fig. 3.** Amino acid sequence comparison of a short stretch of the NS3 protein that has been amplified as described in the text. The sequence domain showing differences is reported in the alignment. cc, Carrion crow; ng, northern goshawk. Mutated amino acids are indicated in red and position is indicated with a triangle (▲).

mice brains were processed for immunohistochemistry (IHC). IHC was performed using rabbit polyclonal antibody (ab22070, Abcam, UK) diluted 1:250. Tissue sections were heat-treated for antigen retrieval (121 °C for 8 min in 0.01 M citrate buffer, pH 6.0) and immune reactions were revealed using a peroxidase technique (Envision Plus kit, Denmark). Positive and negative controls were included in all panels of IHC reactions. The 423-bp fragment of NS3 amplified by nested RT-PCR [7] was purified and used for direct sequencing in both directions using internal primers as described previously [9]. Raw sequence data were assembled and translated into amino-acid sequences (Vector NTI, USA). Consensus sequences were aligned with other WNV lineage 2 NS3 sequences including isolates from 2004 in Hungary (accession no. DQ116961), 2010 in Greece (accession no. HQ537483) and Italy [9] with ClustalW [10].

During necropsy, no significant gross lesions were observed. The WNV RNA detected in the tissues of the three wild birds was identified as WNV lineage 2. Two of the three strains were successfully propagated *in vitro*. The strains isolated and propagated were named ccOlbia/IT2012 and ngUsellus/IT2012, respectively. All mice inoculated showed severe neurological symptoms (ataxia, seizures, prostration) commencing 5 days' post-inoculation and were humanely euthanized. Large amounts of viral antigens were observed in the cerebral cortex and cerebellum of the infected mice (Fig. 2). According to the partial NS3 sequence analyses, the Sardinian strains showed a high level of similarity at both the nucleotide and amino-acid levels. The only difference observed was in the strains detected in the tissue of the two carrion crows (ccSardara/IT2012, ccOlbia/IT2012). These strains showed histidine instead of glutamine at position 245 of NS3. This mutation also distinguished these strains from the homologous sequences of the other WNV lineage 2 strains included in the sequence comparison (Fig. 3). Compared to the Greek isolate, all these strains showed histidine at position 249 instead of proline.

In 2011, at the end of the season, a WNV lineage 2 strain was reported to be responsible for a human case in Ancona, central Italy, and for a human fatality in Olbia [2, 11]. The present study reports for the first time the circulation, spread, and possibly the establishment of an enzootic cycle of lineage 2 strains in Sardinia. These were found in tissue of birds collected during the activities of the national surveillance programme. As the first carrion crow was culled in the same area as the 2011 human case, it may be indicative of its environmental and overwintering adaptation. Unfortunately, it was not possible to make any sequence comparison since the NS3 sequence of the 2011 human strain is not yet available. With all due caution, induced by the limited NS3 fragment sequenced, the sequence analysis suggests a common origin for the Sardinian and Hungarian isolates, thereby implying an important role for short-range migratory birds in the spread of WNV in Italy during their routes from central Europe – where lineage 2 is endemic – to southern Europe. The NS3 gene encodes for the helicase enzyme which displays various functions in genome replication [12]. Proline replacing threonine at position 249 of the WNV-NS3 helicase has been suggested to increase the virulence of WNV lineage 1 in birds in North America [13]. Even if recent European studies do not support this theory

[14, 15], a proline was present at position 249 of the NS3 encoding gene of the Greek isolate, while the Hungarian and Italian strains have histidine at this position. Whether this substitution is responsible for the higher virulence of the Greek strain has not yet been established [16]. Similarly, the role of histidine rather than glutamine at position 245 found in two of the three Sardinian isolates detected in this study is not clear. It may potentially represent a host specific marker as it was found in the two crows and not in the northern goshawk. Further studies regarding host adaptive markers are therefore warranted. According to these findings WNV lineage 2 strains, which have been proven to be pathogenic for humans, have adapted and spread in Sardinia enhancing the risk of new infections in the region. Moreover, these strains have expanded into areas where WNV lineage 1 and Usutu virus strains have circulated/are circulating [9] with the possible occurrence of homologous or heterologous recombination which may affect the diagnosis, virulence and transmission of these strains.

#### ACKNOWLEDGEMENTS

We thank all colleagues for their assistance in field and laboratory aspects of this report. Funding was provided by the Italian Ministry of Health.

#### DECLARATION OF INTEREST

None.

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