Lack of detection of West Nile virus in an islander population of chelonians during a West Nile virus outbreak

Nicola Di Girolamo[1*], Paolo Selleri[1], Annapia Di Gennaro[2], Marco Maldera[3], Giordano Nardini[4], Marco Muzzeddu[5], Francesco C. Origgi[6] & Giovanni Savini[2]

[1] *Clinica per Animali Esotici, Centro Veterinario Specialistico, Via Sandro Giovannini 53, Roma, Italy.*
[2] *Virology Department, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, Teramo, Italy.*
[3] *University of Rome La Sapienza, Rome, Italy.*
[4] *Clinica Veterinaria Modena Sud, Piazza dei Tintori 1, Spilamberto (MO), Italy.*
[5] *Wildlife rehabilitation center Bonassai, Sassari, Italy.*
[6] *Centre for Fish and Wildlife 5 Health (FIWI), Vetsuisse Faculty, University of Bern, Bern, Switzerland.*

** Corresponding author at: Clinica per Animali Esotici, Centro Veterinario Specialistico, Via Sandro Giovanninni 53, Roma, Italy. Tel.: +39 3292003570, e-mail: nicoladiggi@gmail.com.*

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Summary
In 2011, several outbreaks of West Nile disease occurred in Sardinia (Italy). The region hosts several chelonian species. Because of the increasing concern on the potential role that ectotherms may play in the ecology of West Nile virus (WNV), in October 2011 blood samples were collected from 41 endemic Sardinian chelonians and tested for the presence of active WNV infection or neutralizing antibodies by real time polymerase chain reaction (RT-PCR) and serumneutralisation, respectively. Neither WNV neutralising antibodies (0%; 95% CI: 0-8.4%) nor WNV RNA (0%; 95% CI: 0-6.8%) were found in the tested samples. According to the results of this screening survey, it is unlikely that chelonians are involved in the epidemiology of the 2011 WNV outbreaks in Sardinia.

Mancato rinvenimento di West Nile virus in una popolazione isolana di cheloni durante una epidemia

Parole chiave
Cheloni,
Rettili,
Sardegna,
Testuggini,
Tartarughe,
West Nile Virus,
Zoonosi.

Riassunto
Nel 2011 si sono verificate diverse epidemie della febbre del Nilo occidentale in Sardegna (Italia). Nella regione sono endemiche varie specie di cheloni. Visto la delimitazione delle specie isolane e l'aumento dell'attenzione riguardo il potenziale ruolo degli ectotermi nell'ecologia del virus della West Nile (WNV), abbiamo investigato la presenza di WNV in cheloni selvatici. Con questa finalità ad ottobre 2011 sono stati collezionati campioni di sangue da 41 cheloni selvatici. I campioni sono stati testati per la presenza di infezione attiva da WNV o per presenza di anticorpi, tramite real time PCR e sieroneutralizzazione. Nei campioni testati non venivano riscontrati né anticorpi neutralizzanti il WNV (0%; 95% CI: 0-8.4%) né l'RNA del WNV (0%; 95% CI: 0-6.8%). Basandosi sui risultati di questo campionamento, è improbabile che i cheloni siano coinvolti nell'epidemie di WNV in Sardegna.

West Nile virus (WNV) is a *Flavivirus*, maintained worldwide through an enzootic cycle involving primarily avian hosts and mosquito vectors (Mukhopadhyay *et al.* 2003).

In 2011, an outbreak of WN disease (WND) occurred in the island of Sardinia (Italy). Of the 273 horses living in the province of Oristano, 89 were infected by WNV lineage 1 strain (IZSAM-CESME 2011, Spissu *et al.* 2013, Monaco *et al.* 2015). Among them, 49 showed neurological signs such as ataxia, paresis, paralysis, hyperesthesia, muscle tremors, seizures, or fever. Nine of them died or were euthanized (IZSAM-CESME 2011, Spissu *et al.* 2013, Monaco *et al.* 2015). Six human patients suffered the neuroinvasive form of the disease and 4 of them died (Magurano *et al.* 2012). In the following year, WNV lineage 2 strain was detected in Sardinian wild birds (Savini *et al.* 2013).

Besides endotherms, other animals may play a role in the ecology of WNV (Ariel 2011; Marschang 2011). Recently, alligators have been found to act as possible WNV amplifiers (Klenk *et al.* 2004). Furthermore, antibodies to WNV have been detected in a Caspian turtle (*Clemmys caspica*) (Nir *et al.* 1969) and WNV has been isolated from farmed and wild crocodiles (Steinman *et al.* 2003, Machain-Williams *et al.* 2013). Sardinia hosts a rich and distinctive herpetofauna, including 5 chelonian species (Salvi and Bombi 2010); chelonians are generally the larger reptile species in the island (Bernini *et al.* 2006).

While venipuncture is challenging in small reptiles, venous access in adult chelonians is easy and the volume of blood required for virology testing may be safely harvested (Avery and Vitt 1984, Andreani *et al.* 2014). In addition, chelonians are generally over-represented in wildlife care facilities as compared to other reptiles (Rivas *et al.* 2014). Given these reasons, we retained chelonians to be the ideal ectotherms to screen for WNV in Sardinia.

To our knowledge, chelonians have never been screened for the presence of WNV during a WNV outbreak. The aim of the present survey, therefore, was to screen Sardinian resident chelonians for the presence of active WNV infection and/or neutralizing antibodies.

In October 2011, blood samples were collected from endemic Sardinian chelonians. A minimum estimated sample size of 21 individuals was calculated based on the previous year seroprevalence of WNV in horses (IZSAM-CESME 2008, IZSAM-CESME 2010) with 80% of power and 95% confidence level.

The sampled chelonians were from 2 centres for fauna rehabilitation located in the Northern and Southern part of the island, respectively. Chelonians living in these centres have been rescued from different sites of the island including those with WNV circulation (IZSAM-CESME 2011). The animals were manually restrained and from each animal 1.5 to 2 milliliters of blood were collected from the jugular vein using a sterile syringe and a 25-gauge needle. Blood was aliquoted into glass tubes containing lithium-heparin. Plasma was separated by centrifugation, placed into microcentrifuge tubes, and immediately frozen in liquid nitrogen at -196°C. Blood cells were stored with 0.5 ml of RNA later (Ambion, Austin, TX, USA) at -20°C. Blood cells and plasma samples were transported to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Italy, the National and OIE Reference Laboratory for West Nile Fever.

Plasma was heat inactivated for 30 minutes at 56°C. Medium was added to the wells of a sterile 96-well microtitre plate as follows: 40 µl per well from A1 to A8, 25 µl in the wells for the virus control and 50 µl in the wells for the control of cells; 10 µl of each undiluted serum to be tested and negative and positive control sera were added to the first wells of the row A. Dilutions of sera were conducted in the microplates as follows: the first dilution wells of the sera were mixed by pipetting in and out at least 8 times, 25 µl were then transferred from 1 row to the next one, until the last one was reached. Twenty-five µl were discarded from the last row (H).

Stock reference WNV strain (lineage 1) was stored in microtubes at - 80°C. One tube was thawed rapidly under cold running water, and placed in melting ice. One dilution from this tube was prepared in order to obtain 100 $TCID_{50}$ (Tissue culture infectious dose which will infect 50% of the cell monolayer) in 25 µl and were added to each serum-filled well. For virus control, starting from 100 $TCID_{50}$ 4 tenfold dilutions were prepared. The microplate was incubated at 37°C in a humid incubator with 5% CO_2 for 1 hour. After trypsin treatment, the cells of a confluent culture of 3-day-old VERO cells were resuspended in Medium supplemented with 10% Foetal Calf Serum to obtain a 3×10^5 cells/ml suspension. Fifty µl of the cell suspension were successively added to each well. The microplate was incubated at 37°C in a humid incubator with 5% CO_2. The plate was monitored everyday under inverted microscope and when the virus control showed cytopathic effect in the wells C9-C12, the test was read (3-7 days). Results were considered if the positive control confirmed the known titre (± 1 log), the negative serum showed cytopathic effect from the first row and the cell monolayer in the cell control wells was healthy. A serum sample was considered positive when 1/10 dilution was able to neutralise 90% of virus activity.

Blood cell samples were screened for the presence of WNV. RNA was extracted and amplified by a WNV-specific real-time polymerase chain reaction (RT-PCR) able to detect lineages 1 and 2 (Eiden *et al.* 2010).

Forty-one free-ranging chelonians were sampled, including n = 8 Hermann's tortoises (*Testudo hermanni*), n = 21 marginated tortoises (*Testudo marginata*), n = 8 spur-thighed tortoises (*Testudo graeca*), n = 4 red-eared sliders (*Trachemys scripta elegans*). From those specimens, 41 blood cell samples and 33 plasma samples were analysed. Eight plasma samples were excluded from the analysis due to a cold chain break.

Neither WNV neutralising antibodies (0%; 95% CI: 0-8.4%) nor WNV RNA (0%; 95% CI: 0-6.8%) were found in the plasma or blood cell samples, respectively.

According to the results of this screening survey, it appears that chelonians are unlikely involved in the epidemiology of WNV. Sardinia was selected among other possible sites because of the abundant presence of endemic chelonians. More in details, 5 species of the order Testudines may be found in the wild (*Testudines graeca, Testudines hermanni, Testudines marginata, Testudines scripta* and *Emys orbicularis*) (Salvi and Bombi 2011).

Previously, WNV has been isolated from farmed American alligators (*Alligator mississippiensis*) in Georgia (Miller *et al.* 2003), Florida (Jacobson *et al.* 2005a) and Louisiana (Nevarez *et al.* 2005) and from crocodiles in Israel (Steinman *et al.* 2003). Free-ranging alligators presented WNV antibodies in Florida (Jacobson *et al.* 2005b), but not in the neighbouring Louisiana (McNew *et al.* 2007). Interestingly, alligators experimentally infected with WNV were found to have adequate viraemia levels (high and long-lasting titer) for viral transmission by mosquitoes (Klenk *et al.* 2004). In addition, WNV has been detected in mosquitoes associated with alligator farms and in alligator blood detected in mosquitoes acting as WNV vectors (Unlu *et al.* 2010).

There are four possible explanations of our findings:

1. chelonians do not get infected with WNV for some physiological reason. Although in a serosurvey conducted in Israel from 1965 to 1966, antibodies to WNV were detected in a Caspian turtle (*Clemmys caspica*) (Nir *et al.* 1969), which is to our knowledge the only report of WNV in chelonians. In fact, Klenk and Komar (Klenk and Komar 2003) found that red-eared sliders subcutaneously inoculated with WNV do not develop viraemia above the detection threshold of $10^{1.7}$ pfu/ml of serum;
2. chelonians do not get bitten by mosquitoes, which are the main vector of the disease. Mosquitoes feed blood meals from reptiles, including chelonians. For example, alligator blood was detected in 6 species of mosquitoes, including *Culex quinquefasciatus* and *Culex nigripalpus* (Unlu *et al.* 2010). Eleven percent of mosquitoes sampled in the Galapagos Islands fed on terrestrial chelonians (*Geochelone nigra*) (Bataille *et al.* 2009), while the 3% of mosquitoes sampled in a survey conducted in Alabama fed on semi-aquatic chelonians (*Terrapene carolina* and *Trachemys scripta*) (Cupp *et al.* 2004);
3. the chelonians sampled in the present survey did not yet seroconvert at the time of sampling (Klenk and Komar 2003, Klenk *et al.* 2004). In this third scenario, the lack of RNA retrieval from the erythrocytes excluded the chance of a current infection. These hypotheses should be considered with caution as the cited studies on which they rely were performed on different reptile species;
4. a final hypothesis is that none of the chelonian sampled in the present survey was in strict contact with horses, and therefore the negative results of the study were secondary to sampling bias.

Even though further investigations are needed to exclude the possibility of WNV infection in chelonians, it seems unlikely that wild chelonians played any epidemiological role in the 2011 WNV outbreak in Sardinia.

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