Title: Are tree squirrels involved in the circulation of flaviviruses in Italy?

Short Running title: Flavivirus exposure in squirrels

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Summary

West Nile virus (WNV), Usutu virus (USUV) and Tick-borne encephalitis virus (TBEV) are emerging zoonotic flaviviruses (Family *Flaviviridae*), which have circulated in Europe in the past decade. A cross-sectional study was conducted to assess exposure to these antigenically-related flaviviruses in eastern gray squirrels (*Sciurus carolinensis*) in Italy. Seventeen out of 158 (10.8%; $CI_{95\%}$: 5.9-15.6) squirrels' sera tested through bELISA had antibodies against flaviviruses. Specific neutralizing antibodies to WNV, USUV and TBEV were detected by virus neutralization tests. Our results indicate that tree squirrels are exposed to *Culex* and tick-borne zoonotic flaviviruses in Italy. Moreover, this study shows for the first time USUV and TBEV exposure in gray squirrels, broadening the host range reported for these viruses. Even though further studies are needed to define the real role of tree squirrels in the epidemiology of flaviviruses in Europe, this study highlights that serology could be an effective approach for future investigations aimed at broadening our knowledge about the species exposed to these zoonotic infections.

Keywords: Flavivirus; West Nile virus; Usutu virus; Tick-borne encephalitis virus; squirrels; zoonoses

Introduction

Most flaviruses (genus *Flavivirus,* Family *Flaviviridae*) are emerging or re-emerging vector-borne zoonotic pathogens. Among them, West Nile virus (WNV), Usutu virus (USUV) and Tick-borne encephalitis virus (TBEV) have circulated endemically in European countries in the last decade, raising concerns regarding both public and animal health (Beck et al., 2013). Consequently, integrated human, veterinary and vector surveillance systems for flaviviruses have been implemented in several European countries (Gossner et al., 2017). WNV and USUV belong to the mosquito-borne flavivirus group and are generally maintained in an enzootic life-cycle involving ornithophilic mosquitoes (mostly genus *Culex*) as competent vectors and wild birds as main reservoir hosts. Even though mammals are susceptible to infection by these flaviviruses, most species are considered incidental or dead-end hosts, as they typically show a short-term and low-level viremia that prevents transmission to competent vectors (Root, 2013). TBEV is the most relevant zoonotic virus within the tick-borne flavivirus group in Europe. Its epidemiological cycle is maintained by small rodents as reservoirs and hard ticks (genu*s Ixod*es) as vectors.

Previous studies have documented that squirrels are exposed to vector-borne flaviviruses (reviewed in Root, 2013; Demina et al., 2017). These arboreal rodents do not appear to play a major role as amplifying hosts, but contrary to other mammals, WNV infection in tree squirrels (mostly genus *Sciurus*) has been shown to reach sufficient viremia to infect competent mosquito species and the virus has been isolated from fecal and urine samples in experimentally infected animals (Root et al., 2006; Gómez et al., 2008; Platt et al., 2008; Tiawsirisup et al., 2010). Furthermore, North American populations of several tree squirrel species had high seroprevalence to WNV, with several individuals showing evident clinical signs of disease (Root et al., 2005; Padgett et al., 2007; Bisanzio et al., 2015). Because tree squirrels share habitats with wild birds and *Culex* mosquitoes, frequently inhabit urban and periurban areas, can reach high densities and have small home-ranges, in North America these species have been proposed as useful sentinels providing early warning of WNV circulation (Gómez et al., 2008; Root, 2013; Bisanzio et al., 2015). In Europe, conditions are sensibly different since the epidemiology of flaviviruses is different and the only native tree squirrel species, the Eurasian red squirrel (*Sciurus vulgaris*), lives at low densities and usually avoids heavily anthropized areas. However, other alien squirrel species have been introduced in the continent. In particular, the eastern gray squirrel (*S. carolinensis*) is the most abundant and widespread alien squirrel in Europe, having been introduced into the British Isles since the second half of the XIX century and in Italy in 1948 (Bertolino et al., 2014). Distribution of this species in the Italian peninsula is fragmented, with two main populations established in the northwestern part of the country, over an area of about 3500 km^2 in the Po plain (Bertolino et al., 2014). Gray squirrels in Italy are currently being culled within invasive species control programs. However, even though this rodent is among those North American species that show high exposure to WNV in their native range (Root et al., 2005; Bisanzio et al., 2015), there is no information about their role in the epidemiology of flaviviruses in the European range.

Since surveillance programs and early warning systems toward WNV, USUV and TBEV targeting human, animals and vectors have revealed recurrent circulation of these viruses in northern Italy (e.g. Rezza et al., 2015; Rizzo et al., 2016), the goal of this study was to determine the exposure to these flaviviruses in alien gray squirrel populations introduced in the area.

Materials and Methods

A total of 158 gray squirrels from 13 populations located in northern Italy (7 sites in Piedmont and 6 in Lombardy region, Fig 1) were sampled monthly between 2011 and 2013. The sample set was heterogeneous for host sex, age class (i.e. adult or subadult), season and year of collection. Blood samples were collected through heart-puncture in specimens culled within a population control program (LIFE EC-SQUARE), in accordance with EC directives and with local laws and regulations (see Romeo et al., 2014a for details on field procedures). Blood samples were centrifuged and sera were stored at -20 C° until analysis.

Sera were screened using a commercial blocking ELISA (bELISA) (10.WNV.K3 INGEZIM West Nile COMPAC®, Ingenasa, Madrid, Spain) which detects antibodies against one epitope of the envelope protein domain III of the Japanese encephalitis serocomplex (genus *Flavivirus*) (Sotelo et al., 2011). The assays were performed according to the manufacturers' instructions. Results were expressed as a percentage of inhibition (PI) calculated using the optical density (OD) of a sample and the mean OD of the negative control (NC) of the kit as follows: $PI = 100-[(OD_{sample}/OD_{NC})x100]$. According to the instructions of the kit, samples with PI values >40% were considered positive, those with PI values <30% were considered negative, and those with PI values between 30% and 40% were considered doubtful. The bELISA was used as a serological screening tool and bELISA-positive and doubtful sera were then tested by virus neutralization test (VNT) for the detection of specific neutralizing antibodies against WNV (Is98 strain), USUV (It12 strain) and TBEV (Hypr strain) according to World Organisation for Animal Health guidelines (OIE, 2013). Sera that showed neutralization at dilutions ≥ 10 (WNV, USUV) and 20 (TBEV) were considered positive. The neutralizing immune response observed was considered specific when VNT titers for a given virus were at least fourfold higher than titers obtained for the other two viruses. The effect of independent variables (sex, age class, region, season and year) on seropositivity to flaviviruses was investigated through logistic regression, applying Firth's penalized maximum likelihood method to cope with low prevalences and quasi-separation of data (Heinze and Schemper, 2002). The fit and the discriminatory capability of the model were assessed through Hosmer and Lemeshow test (X^2_8=8.7; p=0.37) and the Receiver Operator Curve (Area Under the Curve=0.82), respectively. All the analyses were carried out using PROC LOGISTIC in SAS® 9.4 Software (Copyright © 2012 SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Antibodies against flaviviruses were detected in 17 out of 158 gray squirrels (10.8%; 95% Confidence Interval: 5.9-15.6) tested by bELISA. Specific neutralizing antibodies against WNV, USUV and TBEV were then confirmed by VNT in one, five and three of the 17 bELISA-positive squirrels, respectively. One animal was positive to both USUV and TBEV neutralizing antibodies, with titer differences ≤ 2-fold. Although this last finding may be related to VNT cross-reactivity among USUV and TBEV, co-infection by both viruses cannot be ruled out. The seroprevalence in squirrels was 0.6% for WNV and, considering the possible co-occurrence, ranged between 3.2 and 3.8% for USUV and 1.9 and 2.5% for TBEV. The remaining seven bELISA-positive sera were negative against the three viruses tested using VNT, suggesting exposure to other, cross-related flaviviruses. Other than WNV, USUV and TBEV, only insect-specific flaviviruses have been isolated in mosquitoes in Northern Italy (Rizzo et al., 2014; Grisenti et al., 2015). However, the circulation of other flaviviruses among wild mammals in Italy cannot be excluded (Cosseddu et al., 2017). In this respect, several flaviviruses, including Meaban virus, Louping ill virus and Bagaza virus have been detected in other European countries in the last few years (Beck et al., 2013; García-Bocanegra et al., 2013;

Arnal et al., 2014). Further investigations through molecular methods would help to disclose the matter and detect other flaviviruses circulating in the study area.

We detected a single VNT-positive animal to WNV and no outbreaks were reported in the study area in 2011 (CESME, 2017), when the seropositive squirrel was sampled. Although a false positive result cannot be completely ruled out, this finding may also indicate a limited circulation of WNV in the study area in 2011. Our results also highlight for the first time natural USUV exposure in a squirrel species, broadening the host range reported for this zoonotic flavivirus (Gaibani and Rossini, 2017). Finally, the present study represents the first report of natural TBEV exposure in an arboreal rodent as the virus had previously been isolated only from long-tailed ground squirrels (*Spermophilus undulatus*) and from experimentally infected dormice (*Glis glis*) (Kozuch et al., 1963; Demina et al., 2017). Seroprevalence to TBEV observed in gray squirrels in the present study is lower than prevalence reported in rodents and goats in northeastern Italy (Rizzoli et al., 2007). This was not surprising, since both gray and red squirrels (*S. vulgaris*) over the same range in northwestern Italy are rarely infested by ticks (Romeo et al., 2014a).

Six out of the 13 (46.1%) sampling sites presented at least one seropositive squirrel to flaviviruses detected by bELISA (Fig 1). Seropositivity to flaviviruses in squirrels varied across regions (X^2_1=7.3, p=0.007): it was significantly higher in Piedmont (16.8%; 15/89) compared to Lombardy (2.9%; 2/69). Moreover, all squirrels that showed VNT-positive results for either WNV, USUV or TBEV were trapped in Piedmont. In this respect, our results contrast with the higher circulation of WNV and USUV detected in both humans and competent vectors in Lombardy region during the study period (Chiari et al., 2015; Rizzo et al., 2016; Calzolari et al., 2017; CESME, 2017; Mancini et al., 2017). The regional difference in seroprevalence observed in our study is likely related to habitat differences between the distribution range of grey squirrels in the two regions. Most sampling sites in Piedmont were woodlands fragments surrounded by open fields and located in flat, humid areas. Conversely, most sampling sites in Lombardy were larger woods with a drier climate, which are less favorable habitats for the development of mosquito vectors. Indeed, most of flavivirus outbreaks reported in Lombardy region were located further to the south than our study areas and outside of grey squirrels' introduction range (e.g. Chiari et al., 2015; Rovida et al., 2015; Calzolari et al., 2017). Seropositivity to flaviviruses significantly varied also across years (X^2_2=6.7, p=0.04), with a higher seroprevalence in squirrels sampled in 2011 (29.0%; 9/31), compared to 2012 (6.9%; 5/72) and 2013 (5.4%;

3/55). USUV-specific neutralizing antibodies were observed in all the three sampled years, while seropositivity to WNV and TBEV was only found in 2011. The presence of antibodies against WNV and USUV in young animals trapped in Piedmont indicates circulation of these viruses in 2011 and 2012, respectively, which is consistent with serological data from wild birds over the same geographical area (Llopis et al., 2015). However, WNV cases in the region were reported for the first time in horses and birds only in 2014; while USUV cases in horses were detected already in 2010, suggesting a more intense circulation of USUV compared to WNV (Calzolari et al., 2017; CESME, 2017), which may explain the higher seroprevalence to USUV observed in tree squirrels (CESME, 2017). Host-related factors (i.e. sex and age) and seasons had no effect on seropositivity to flavivirus (all $p>0.05$). Nevertheless, our results should be carefully interpreted because of the limited number of analyzed animals.

In conclusion, our findings indicate that gray squirrels are exposed to *Culex* and tick-borne flaviviruses, particularly WNV, USUV and TBEV in Italy. Even though this species does not appear to play a major role in the epidemiology of flaviviruses, our results, as well as previous epidemiological data from North America and experimental infections, suggest that this species might be involved in the circulation of these zoonotic flaviviruses. Finally, our findings highlight how invasive alien species should not be considered only as carriers of new pathogens, but also as potential reservoirs for local diseases (Hatcher et al., 2012). The risk of underestimating the epidemiological impact of introduced hosts might be even greater for those species, such as squirrels, for which only a limited number of diseases is known (Romeo et al., 2014a; 2014b). Therefore, a deeper understanding of the mechanisms driving the spatio-temporal variability observed in WNV, USUV or TBEV circulation is essential to define the true role of squirrels in the epidemiology of flaviviruses in Europe.

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Conflict of interest statement

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figure legend

Figure 1. Map of northwestern Italy showing sites where gray squirrels were examined for Flaviviruses' exposure. Black and white dots indicate positive and negative sites for the presence of flaviviruses detected by bELISA, respectively. When positive, results of virus neutralization tests are specified above dots. Line patterns represent gray squirrels' distribution in 2015.