

Investigation into Usutu and West Nile viruses in ticks from wild birds in Northwestern Italy, 2012-2014

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SUMMARY

To assess the potential role of ticks as carriers of West Nile virus (WNV) and Usutu virus (USUV), we tested 1721 ticks from 379 wild birds in Northwestern Italy between 2012 and 2014. Ticks were analyzed in pools using a pan-flavivirus real-time RT-PCR and positive pools were subjected to RT-PCR for USUV and WNV genome detection. All the tested samples resulted negative, suggesting that *Ixodes* spp. ticks, at least in our study area, are not competent vectors and not even exploitable sentinels for USUV and WNV.

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Usutu virus (USUV) and West Nile virus (WNV) are emerging pathogens belonging to the family Flaviviridae, genus *Flavivirus*. Their natural cycles involve ornithophilic mosquitoes and wild bird species (Hubálek 2008). Although mosquitoes are considered the primary vectors for WNV, evidence of tick-borne transmission has been documented (Lawrie 2004).

We conducted a study to investigate the potential role of ticks as carriers and spreaders of WNV and USUV. Birds were captured using mist nets in the frame of ringing campaigns in the Scrivia river valley (province of Alessandria, Piedmont region). Between March 2012 and November 2014, ticks were systematically collected from migratory and resident birds, removed with tweezers, preserved in 70% ethanol and identified using morphological keys (Manilla 1998, Cringoli *et al.*, 2005). RNA was extracted from ticks grouped in pools according to species, stage, host and season, with a maximum number of 20 ticks per pool. All *Ixodes* spp. ticks were analyzed by PCR. The presence of flaviviruses was investigated by using a pan-flavivirus real-time polymerase chain reaction (Johnson *et al.*, 2010). Positive pools were subjected to one-step reverse transcription-PCR (RT-PCR) assays with USUV and WNV specific primers (Weissenböck *et al.*, 2004; Chaskopoulou *et al.*, 2011) and sequencing analysis.

Overall, 1723 ticks were collected on 381 birds belonging to 14 species; of these, 7 were short-distance and 7 long-distance migrants (Table 1). The large majority of ticks (91.9%) were collected on blackbirds (*Turdus merula*). Ticks were identified as *Ixodes ricinus* (96.1%, 1 adult, 1478 nymphs, 177 larvae), *Ixodes frontalis* (2 adults, 2 nymphs), *Ixodes acuminatus* (3 adults) and *Hyalomma* spp. (2 nymphs); 58 *Ixodes* spp. specimens (51 nymphs, 7 larvae) could only be identified at genus level as they were

damaged. Blackbirds (n=300) hosted 70.7% of collected larvae and 94.6% of nymphs. A total of 159 pools were analyzed in real time RT-PCR for the presence of flaviviruses. Nineteen pools were positive with low mean Ct values (30,6±2.7). The flavivirus positive pools were then tested by specific PCR for USUV and WNV and resulted negative. Sequencing of pan-flavivirus positive samples was attempted but we did not obtain a readable chromatogram, probably due to the low virus copy numbers.

Results of this study support previous observations that blackbirds play an important role in the dispersal of immature stages of *I. ricinus* ticks and, in perspective, of their associated pathogens (Mannelli *et al.*, 2005).

WNV has been repeatedly isolated from ticks of Ixodidae and Argasidae families in Russia, Israel and Kenya (L'vov *et al.*, 2002; Lwande *et al.*, 2014; Mumcuoglu *et al.*, 2005), and some studies suggested that selected tick species might play a role in the circulation of WNV (Anderson *et al.*, 2003). Moreover, vector competence studies have shown the capacity for some tick species (*Ornithodoros moubata*, *Ornithodoros erraticus*, *Ornithodoros maritimus*, *Argas arboreus* and *Hyalomma marginatum*) to acquire WNV from infected animals and subsequently to transmit the virus to uninfected hosts (Vermeil *et al.*, 1960; Abbassy *et al.*, 1993; Lawrie *et al.*, 2004; Formosinho and Santos-Silva, 2006), suggesting a potential reservoir role of ticks for WNV. However, whether or not Ixodid ticks are vectors of WNV has not been investigated in depth (Lawrie *et al.*, 2004).

Even if the circulation of USUV and WNV has been reported in wild birds and mosquitoes in Northwestern Italy, including our study area (Victoriano *et al.*, 2015a), none of our tick pools tested PCR positive for the two viruses. We may speculate on the reasons for the negative results. Firstly, our previous serological study of USUV and WNV involving more than 900 birds of 88 species and 14 different orders in the Scrivia river valley highlighted a low seroprevalence of both viruses (Victoriano *et al.*, 2015b). Moreover, only few mosquito pools were found USUV and WNV positive in Alessandria province in 2013-2015 (http://sorveglianza.izs.it/emergenze/west_nile/emergenze.html). Accordingly, the negative results found in ticks may simply mirror the limited circulation of both viruses in the study

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Table 1 - Number of ticks found per bird species. Migratory behavior of bird species: long distance migrants (T), short distance migrants (M) and residents (R).

Bird species	Migratory behavior	No of infested birds	No of collected ticks		
			Larvae	Nymphs	Adults
<i>Acrocephalus scirpaceus</i>	T	1	-	1	-
<i>Erithacus rubecula</i>	M	10	9	5	-
<i>Fringilla coelebs</i>	M	1	1	-	-
<i>Garrulus glandarius</i>	R-M	4	-	7	-
<i>Hippolais polyglotta</i>	T	2	-	2	-
<i>Luscinia megarhynchos</i>	T	40	36	45	-
<i>Parus major</i>	R-M	9	3	7	-
<i>Phoenicurus phoenicurus</i>	T	2	3	2	-
<i>Phylloscopus bonelli</i>	T	1	-	1	-
<i>Sturnus vulgaris</i>	M	3	1	4	1
<i>Sylvia atricapilla</i>	R-T	3	1	5	-
<i>Sylvia communis</i>	T	2	-	2	-
<i>Turdus merula</i>	M	300	130	1450	5
<i>Turdus philomelos</i>	M	2	-	2	-
Total		381	184	1533	6

area. Secondly, Lawrie *et al.* (2004) reported that *I. ricinus* ticks can be infected after feeding upon WNV viremic hosts, but are unable to maintain the virus. Indeed, they did not find any evidence of WNV infection in nymphs one month after engorgement, suggesting that *I. ricinus* do not support the replication of the virus, and are not competent vectors for WNV. By extrapolation, *I. ricinus* ticks are also unlikely to be competent vectors of USUV, although this hypothesis will need experimental confirmation.

The role of *Ixodes pacificus*, as a possible vector of WNV was investigated in California by Reisen *et al.* (2007). In that experiment, they investigated the ability of the tick to become infected with the WNV NY99 strain while feeding on viremic song sparrows (*Melospiza melodia*), to maintain the infection transstadially, and then to transmit WNV to song sparrows. The study results indicated that *I. pacificus* ticks were not able to transmit WNV experimentally. Interestingly, a second experiment by Anderson *et al.* (2003) demonstrated that *Ixodes scapularis* larvae can acquire WNV from viremic mice but virus titers decreased rapidly in the days following completion of feeding. Larvae were able to pass the virus transstadially, but naive animals fed upon by nymphs did not become infected. Then, vector competence of *I. scapularis* was not demonstrated.

Our study has limitations in that the experiment was conducted in a single ringing station, and basically involved a single dominant tick species. However, Mancini *et al.* (2013) did not detect USUV and WNV in *Hyalomma* nymphs and a few *I. ricinus* immatures from wild birds in

Central Italy. In conclusion, our negative results and the available literature point towards the conclusion that *Ixodes spp.* ticks are not competent vectors and not even exploitable sentinels for WNV and possibly USUV. However, experimental studies focusing on USUV are necessary to confirm this conclusion. Studies in other Italian locations with higher WNV and USUV prevalence are necessary to verify variations in the relative role of different ticks as sentinels or vectors. Moreover, based on our results, further research would be necessary to clarify the presence of other flaviviruses in ticks from Piedmont.

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