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Further circulation of West Nile and Usutu viruses in wild birds in Italy

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ABSTRACT

Usutu virus (USUV) and West Nile virus (WNV) are emerging pathogens that can cause neurological disease in humans. From March 2012 to June 2013, a sero-survey on wild birds was carried out to investigate the circulation of both viruses in Northwest Italy. Samples belonging to 47 different bird species have been collected using a volunteer based network and a wildlife rehabilitation center. Four of 297 serum samples had neutralizing antibodies against USUV ($P = 1.34\%$, IC 95% 0.36–3.4), while 10 of 233 samples tested positive for WNV ($P = 4.29\%$, IC 95% 2.07–7.75). Neutralizing antibodies for WNV were significantly more prevalent ($p < 0.001$) in trans-Saharan migrants ($P = 21\%$, IC 95% 9.55–37.3) than in resident and short-distance birds, but no migratory habit-related differences were found for USUV. Antibodies in resident bird species suggest that both viruses are circulating in NW Italy.

1. Introduction

Usutu virus (USUV) and West Nile virus (WNV) are emerging neuro-pathogenic agents that belong to the Japanese encephalitis virus antigenic complex of the family *Flaviviridae*, genus *Flavivirus*. Both viruses are maintained in the environment through a bird-mosquito life cycle (Hubálek, 2008) whereas mammals including humans are so far regarded as incidental or dead-end hosts. Migratory birds are assumed to have a key role in the amplification and circulation of these viruses (Malkinson and Banet, 2002; Weissenböck et al., 2002). WNV appeared for the first time in Italy in 1998 causing encephalitis in horses in Tuscany (Autorino et al., 2002). Ten years later, WNV reappeared in northern Italy affecting horses and humans (Calistri et al., 2010a,b; Monaco et al., 2010, 2011). In order to monitor and control WNV circulation, a serological, entomological and virological surveillance program for West Nile neuroinvasive disease has been implemented at the national level by the Italian Ministry of Health (Italian Ministry of Health, 2008). Within this framework it was possible to detect viral circulation in birds, mosquitoes and equids (Monaco et al., 2010, 2011; Calzolari et al., 2010; Savini et al., 2012, 2013) and several human cases were reported (Calistri et al., 2010a; Rizzo et al., 2009, 2012; Bagnarelli et al., 2011; Delbue et al., 2014). USUV was first reported in Austria in 2001 when a considerable die-off of Eurasian blackbirds (*Turdus merula*) was observed in and around Vienna, but a recent retrospective analysis of archived samples from death birds in the Tuscany region (Italy) in 1996 provided evidence for an earlier introduction into Europe (Weissenböck et al., 2002, 2013). USUV has been noticed again in the last decade in some regions of Northern Italy by virological and serological methods (Calzolari et al., 2013; Lelli et al., 2008; Savini et al., 2011). In 2009, two cases of human encephalitis associated to USUV infection were reported in Emilia Romagna region (northern Italy) confirming the zoonotic potential of this virus (Cavrini et al., 2009; Pecorari et al., 2009).

By working in collaboration with organizations caring for wildlife the surveillance program may be improved by increasing the number and diversity of samples (Nemeth et al., 2007). Moreover, wildlife rehabilitation centers may greatly enhance and simplify surveillance efforts for avian-related viruses in some areas by concentrating many samples in limited space (Nemeth et al., 2007). Samples collected from free-ranging birds during ringing campaigns may also provide additional epidemiological information (Komar, 2000). In this perspective, we performed a serological investigation within wild birds collected in the Piedmont region in order to investigate the circulation of WNV and USUV viruses. Furthermore, we evaluated how the use of serological investigation from wild birds, obtained by volunteer networks, may integrate the data derived from official surveillance protocols.

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2. Materials and methods

2.1. Study area and sampling sites

The study was carried out in Piedmont, a region of Northwest (NW) Italy (Fig. 1A). From March 2012 to June 2013, 304 blood samples were collected from wild birds belonging to 47 different species (Table 1A and B). Of these, 168 individuals were captured using mist-nets placed in two different ringing stations: Scrivia river Valley ($N = 147$) (province of Alessandria, 44.8087 N, 8.8572 E) and San Genuario marsh reserve ($N = 21$) (province of Vercelli, 45.2175 N, 8.1777 E). These locations were selected based upon the high ecological richness and the abundance of mosquitoes (Pollono et al., 1998). Several bird species often breed in both locations, and, remarkably, the Scrivia river Valley is along one of the main migratory paths between Europe and Africa (Silvano and Boano, 2008). Captured birds were identified according to species, sex and age class (Spina and Volponi, 2008a). Birds were then ringed, sampled and released. Other blood samples ($N = 136$) were made available by the C. A. N. C. ("Centro recupero animali non convenzionali"), a wildlife rehabilitation center at the Department of Veterinary Sciences, University of Turin, which hospitalizes rescued birds from several areas of NW Italy. Samples origin is shown in Fig. 1B.

Birds were classified in one of the following three groups according to their migratory habits, as indicated in Table 1A and B: residents, short-distance migrants and trans-Saharan migrants (Spina and Volponi, 2008a,b). For those species with a mixed behavior, prevalence data were treated independently and attributed each time to one and to the other behavior group.

2.2. Sampling procedure

Blood samples were collected by syringes or capillary tubes for micro-hematocrit from the brachial or jugular veins according to species. The volume of collected blood never exceeded the 1% of body mass (McGuill and Rowan, 1989). Samples were allowed to clot at room temperature and then centrifuged for 10 min at 5600 g for Eppendorf tubes (Eppendorf Srl Milan, Italy) and 5 min at 3500 g for micro-hematocrit tubes. Sera were stored at −20 °C until use.

2.3. Laboratory tests

2.3.1. Virus strains

USUV strain 939/01 isolated from a blackbird in Vienna (Austria) in 2001 and WNV strain Eg-101 were kindly donated by Prof Zdenek Hubalek (Medical Zoology Laboratory, Institute of Vertebrate Biology, Academy of Sciences, Valtice, Czech Republic) and the Unité des Arbovirus et des Fièvres hémorragiques, Institut Pasteur, Paris (France), respectively. The two viruses are routinely used for the diagnostic activities at the Istituto Zooprofilattico Sperimentale of Teramo.

2.4. Serological investigation

A total number of 304 serum samples were tested by serum-neutralization (SN) assay according to a recent protocol developed by our group (Di Gennaro et al., 2014). The small volume of some sera also influenced the diagnostic pipeline. In particular, out of 304 samples, 233 and 297 serum samples were tested for the presence of WNV and USUV neutralizing antibodies, respectively. Of these, 226 samples were tested simultaneously for WNV and USUV. In few cases ($N = 34$) the serological screening for USUV was performed starting at 1:20 dilution of the tested serum.

2.5. Molecular detection of WNV and USUV

Two real-time RT-PCR assays were employed for the molecular detection of WNV (Del Amo et al., 2013) and USUV (Cavrini et al., 2007). Nucleic acids were purified from the blood samples of serological positive birds by means of BioSprint 96 One-For-All Vet Kit (QIAGEN, Germany).

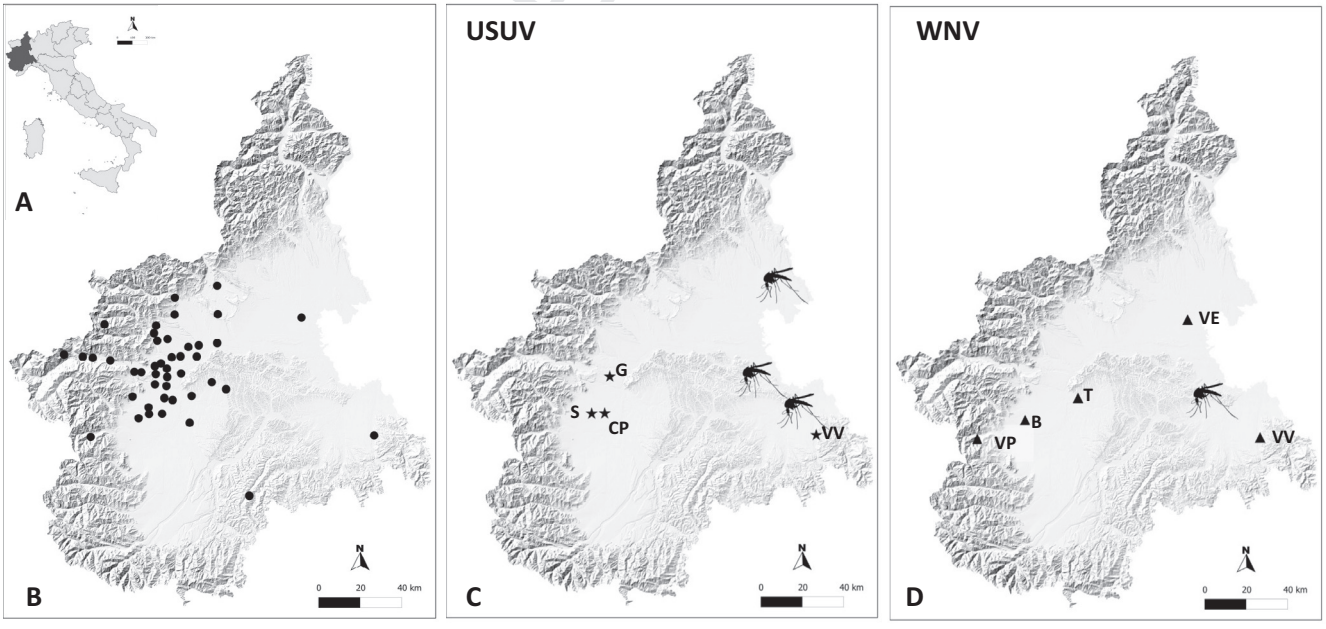

Fig. 1. Map of Italy highlighting the geographical position of Piedmont region (A). Map of Piedmont region showing the geographical origin of the samples (B). Geographical origin of USUV positive samples: municipalities of Villarvernia (VV), Scalenghe (S), Grugliasco (G) and Castagnole Piemonte (CP) (C) and positive samples for WNV: municipalities of Vercelli (VE), Buriasco (B), Villarvernia (VV), Trofarello (T) and Villarpellice (VP) (D). : Geographical location of mosquitoes' pools positive for USUV (B) and WNV (C) RNA demonstrated in 2009/2010, 2012 and 2014.

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