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SURVEY OF FLAVIVIRUSES ON LONG- AND SHORT-DISTANCE MIGRATORY BIRDS IN TRENTINO-ALTO ADIGE (NORTH-EASTERN ITALY) WITH ORAL AND CLOACAL SWABS

GRISENTI M.1,2§, ARNOLDI D.1, RIZZOLLI F.1, GIACOBINI M.2, BERTOLOTTI L.2, RIZZOLI A.1

1Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy.
2Department of Veterinary Sciences - University of Turin, Grugliasco (Torino), Italy. §Corresponding author: Michela Grisenti DMV.

Introduction

West Nile virus (WNV) and Usutu virus (USUV) (genus *Flavivirus*, Flaviviridae family), are emerging as important pathogens. They are maintained in nature by a cycle involving ornithophilic mosquitoes, principally *Culex* spp., and wild birds. In Italy, the first outbreak of WNV occurred in 1998 in Tuscany. Starting from 2008, WNV has been detected every year in animals, mosquitoes, and humans in an increasing number of Italian Regions (Barzon et al., 2013). USUV was historically only detected in tropical and subtropical Africa. The first European cases were confirmed in Italy in 1996 (Weissenböck et al., 2013). In the following years, the virus was detected in birds and/or mosquitoes of several European Countries, with an increasing pathogenicity in European wild birds and humans (Vazquez et al., 2011; Gaibani et al., 2013). Migratory birds have the potential to maintain and spread several *Flaviviruses* moving between Europe, Asia, North Africa (intrapaleartic or short-distance migrants) and southern Africa (long-distance or transaharian migrants) (Pfeffer and Dobler, 2010). Among the non-vectorial transmission routes of these viruses between birds, oral and fecal viral shedding can play an epidemiological role (Komar et al., 2003; Reiter, 2010). There is still a knowledge gap on the role played by the different bird species in the ecology and transmission mechanisms of these viruses. Trentino-Alto Adige (North-eastern Italy) is located on the migratory route of many of the short- and long-distance migratory birds that cross the Alps. Until now, only a silent circulation of WNV and USUV has been detected during 2002 (seroprevalence of 6,09% in forestry workers: Rizzoli and Versini, unpublished results) and during the summer of 2005 (seroconversion in chickens, Rizzoli et al., 2007), but continuous spillover events of both viruses have been reported in the neighbouring regions. The aim of this study was to detect if active virus shedding occurs in migratory birds captured during their seasonal movements.

Materials and methods

Sample collection was carried out in Trentino-Alto Adige region (Figure 1), in autumn 2011 and 2012 (September and October) and spring 2012 (March to May), within the ringing campaigns of the European Union for Bird Ringing (EURING). Oral and cloacal samples were taken from intrapaleartic and transaharian migratory birds using sterile swabs with transport medium AMIES without charcoal, in polypropylene tubes Ø 12×150 mm (Nuova Aptaca S.r.l., Canelli - AT, Italy). Date of capture, species, ring number, age, weight and other morphobiometric parameters were recorded for each individual. After RNA extraction realized with QIAamp® Viral RNA kit (Qiagen, Hilden, Germany), up to 1 µg of RNA was reverse-transcribed according to QiagenQuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). For the screening of Flaviviruses, we used a generic nested RT-PCR that amplifies a region of the NS5 gene, according to (Sanchez-Seco et al., 2005), with modifications (using 5 µl of the cDNA of the first PCR, 5 U of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 40 pmol of each generic Flavivirus primer (Flavi1+, Flavi1-), and 10 nmol of each dNTP). In the nested PCR mix, 1 µl of PCR product from the first reaction was added to 49 µl of reaction mix composed by 1.25 U of HotStarTaq DNA Polymerase, 40 pmol of each primer (Flavi2+, Flavi2-), and 10 nmol for each dNTP. Finally, the products of the nested PCR were analysed by electrophoresis with a 1.5% (w/v%) agarose gel (Sigma-Aldrich, Milan, Italy) and visualized by staining with 0.1% (w/v%) of ethidium bromide.

Results

A total of 43 birds were captured during the autumn of 2011, 176 during spring 2012, and 103 during autumn 2012 (Table 1). Among the 39 species captured, 18 were long-distance migratory, and 21 short-distance migratory species. Oral and cloacal swabs taken from each individual captured all tested negative for *Flaviviruses*. The positive control tested always positive, and the negative one resulted always negative.

Discussion

Flaviviruses transmission dynamics is based on a complex relationship between virus, host, vector species, environmental and climatic factors. In previous studies, the oro-fecal shedding has been found only in some of the bird species (for e.g.: Chvala et al., 2005, 2006; Komar et al., 2002, 2003; Nemeth et al., 2007). Our results further corroborate the results of a previous study carried out in Italy, which found there was no evident oro-faecal shedding of USUV and WNV in the families Fringillidae, Lanidae, Paridae, Muscicapidae, Silvidae, Turdidae, Hirundinidae and Picidae (Lelli et al., 2008). Our results also seem to suggest that families Motacillidae, Prunellidae, Emberizidae, Cuculidae, Egitalidae, Strigidae may not be important shedders. The lack of detection of active virus shedding does not exclude the circulation of these viruses within this region. Their circulation is anyway apparently very limited. Many factors can be involved: the lenght of the shedding with regard to the lenght of the migratory movements, the low density of mosquitoes, the lack of suitable habitat for *Culex* spp., the mountainous orography of the territory and the high avian biodiversity (Grisenti et al., 2013 and references therein).

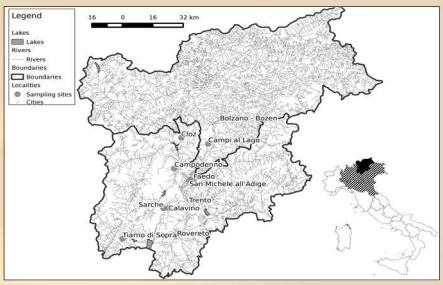

Figure 1: Bird sampling sites. Italian map insert: black area, sampling region of Trentino-Alto Adige; hatched area, neighbouring regions (Veneto, Lombardy, Emilia Romagna, Friuli-Venezia Giulia) with active WNV and USUV circulation.

Bird species		Family	Order	Migratory pattern[a]	2011 Autumn (n)	2012 Spring (n)	2012 Autumn (n)	Total
Scientific name	Common name							
Otus scops	European Scops Owl	Strigidae	Strigiformes	L	-	-	1	1
Cuculus canorus	Cuckoo	Cucilidae	Cuculiformes	L	-	1	-	1
Jynx torquilla	Wryneck	Picidae	Piciformes	L	-	1	-	1
Aegithalos caudatus	Long tailed Tit	Egitalidae	Passeriformes	S	-	2	2	4
Lanius collurio	Red backed Shrike	Lanidae	Passeriformes	L	-	5	1	6
Delichon urbica	House Martin	Irundinidae	Passeriformes	L	-	-	5	5
Emberiza schoeniclus	Reed Bunting	Emberizidae	Passeriformes	S	-	3	-	3
Prunella modularis	Dunnoch	Prunellidae	Passeriformes	S	2	2	-	4
Anthus trivialis	Tree Pipit	Motacillidae	Passeriformes	L	-	-	1	1
Ficedula hypoleuca	Pied Flycatcher	Muscicapidae	Passeriformes	S	-	4	6	10
Muscicapa striata	Spotted Flycatcher	Muscicapidae	Passeriformes	L	-	12	-	12
Sylvia borin	Garden Warbler	Silvidae	Passeriformes	L	-	9	1	10
Sylvia curruca	Lesser Whitethroat	Silvidae	Passeriformes	S	-	-	3	3
Hippolais polyglotta	Melodius Warbler	Silvidae	Passeriformes	L	-	2	-	2
Hippolais icterina	Icterin Warbler	Silvidae	Passeriformes	L	-	2	-	2
Acrocephalus scirpaceus	Reed Warbler	Silvidae	Passeriformes	L	-	15	-	15
Acrocephalus palustris	Marsh Warbler	Silvidae	Passeriformes	L	-	5	-	5
Acrocephalus arundinaceus	Great reed Warbler	Silvidae	Passeriformes	L	-	6	-	6
Sylvia atricapilla	Blackcap	Silvidae	Passeriformes	S	1	23	1	25
Locustella naevia	Grashopper Warbler	Silvidae	Passeriformes	L	-	1	-	1
Sylvia melanocephala	Sardinian Warbler	Silvidae	Passeriformes	S	-	1	-	1
Philloscopus trochilus	Willow Warbler	Silvidae	Passeriformes	L	-	10	5	15
Philloscopus collybita	Chiffchaff	Silvidae	Passeriformes	S	-	9	-	9
Philloscopus sibilatrix	Wood Warbler	Silvidae	Passeriformes	L	-	2	-	2
Periparus ater	Coal Tit	Paridae	Passeriformes	S	-	-	7	7
Parus major	Graet Tit	Paridae	Passeriformes	S	-	1	-	1
Phoenicurus phoenicurus	Redstart	Turdidae	Passeriformes	L	-	2	2	4
Phoenicurus ochruros	Black Redstart	Turdidae	Passeriformes	S	-	-	3	3
Oenanthe oenanthe	Wheatear	Turdidae	Passeriformes	S	-	-	1	1
Turdus merula	Blackbird	Turdidae	Passeriformes	S	6	6	15	27
Erithacus rubecula	Robin	Turdidae	Passeriformes	S	9	33	24	66
Turdus viscivorus	Mistle Thrush	Turdidae	Passeriformes	S	1	-	-	1
Turdus philomenos	Song Thrush	Turdidae	Passeriformes	S	10	9	18	37
Turdus iliacus	Redwing	Turdidae	Passeriformes	S	-	-	1	1
Luscinia megarhynchos	Rufus Nightingale	Turdidae	Passeriformes	L	-	6	-	6
Fringilla coelebs	Chaffinch	Fringillidae	Passeriformes	S	7	2	3	12
Coccothraustes coccothraustes	Hawfinch	Fringillidae	Passeriformes	S	4	2	-	6
Carduelis spinus	Siskin	Fringillidae	Passeriformes	S	1	-	3	4
Fringilla montifringilla	Brambling	Fringillidae	Passeriformes	S	2	-	-	2
Total (n)					**43**	**176**	**103**	**322**

Table 1: Bird species tested in Trentino-Alto Adige in 2011 and 2012 ([a]Each species was classified as intrapaleartic (S) and transaharian (L).

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