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1 **USUTU VIRUS IN ITALY, AN EMERGENCE OR A SILENT INFECTION?**

2 Giovanni Savini¹, Federica Monaco¹, Calogero Terregino², Annapia Di Gennaro¹, Luca Bano², Chiara
3 Pinoni¹, Roberta De Nardi², Paolo Bonilauri³, Monica Pecorari⁴, Luigina Di Gialleonardo¹, Lebara
4 Bonfanti², Andrea Polci¹, Paolo Calistri¹, Rossella Lelli¹

5 ¹ Istituto G. Caporale, Teramo, Italy

6 ² Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy

7 ³ Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Reggio Emilia, Italy

8 ⁴ Department of Diagnostic and Laboratory Services and Legal Medicine, University of Modena and
9 Reggio Emilia, Azienda Ospedaliera Policlinico, Modena, Italy

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21 **Corresponding author:** Giovanni Savini
22 Dept. of Virology
23 National Reference Center for West Nile disease
24 OIE Reference laboratory for Bluetongue
25 Istituto G. Caporale Teramo
26 Via Campo Boario, 64100 - Teramo - Italy
27 Telephone: +390861 332440
28 Fax No. +390861 332251
29 E-mail: g.savini@izs.it

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1 ABSTRACT

2 A two year study (2008-2009) was carried out to monitor the Usutu virus (USUV) circulation in Italy.
3 Sentinel horses and chickens, wild birds and mosquitoes were sampled and tested for the presence of
4 USUV and USUV antibodies within the WND National Surveillance plan. Seroconversion evidenced in
5 sentinel animals proved that in these two years the virus has circulated in Tuscany, Emilia Romagna,
6 Veneto and Friuli Venezia Giulia regions. In Veneto USUV caused a severe blackbird die-off disease
7 involving at least a thousand birds. Eleven viral strains were detected in organs of 9 blackbirds (52.9%)
8 and two magpies (0.5%) originating from Veneto and Emilia Romagna regions. USUV was also
9 detected in a pool of *C. pipiens* caught in Tuscany. According to the alignment of the NS5 partial
10 sequences, no differences between the Italian USUV strains isolated from Veneto, Friuli and Emilia
11 Romagna regions were observed. The Italian North Eastern strain sequences were identical to those of
12 the strain detected in the brain of a human patient and shared a high similarity with the isolates from
13 Vienna and Budapest. Conversely, there were few differences between the Italian strains which
14 circulated in the North Eastern regions and the USUV strain detected in a pool of *C. pipiens* caught in
15 Tuscany. A high degree of similarity at both nucleotide and amino acid level was also found when the
16 full genome sequence of the Italian North Eastern isolate was compared with that of the strains
17 circulating in Europe. The North Eastern Italian strain sequence exhibited 97% identity to the South
18 African reference strain SAAR-1776. The deduced amino acid sequences of the Italian strain differed
19 by 10 and 11 amino-acids from the Budapest and Vienna strains, respectively, and by 28 from the
20 SAAR-1776 strain. According to this study two strains of USUVs are likely to have circulated in Italy
21 between 2008 and 2009. They have developed strategies of adaptation and evolution to spread into
22 new areas and to become established.

23

24 **KEY WORDS:** Chickens, genome sequencing, horse, Italy, NS5, Usutu virus, wild birds

25

1 INTRODUCTION

2 Usutu virus (USUV) is a single strand RNA virus included in the mosquito-borne cluster of the genus
3 *Flavivirus*, within the Family *Flaviviridae*. Together with human pathogens like Japanese encephalitis
4 virus (JEV), Murray Valley encephalitis virus (MVEV), Saint Louis encephalitis virus (SLEV) and West Nile
5 virus (WNV) (Kuno et al., 1998; Bakonyi et al., 2004), it is included in the Japanese encephalitis virus
6 serogroup (Heinz et al., 2000). As all members of this group, the virus is maintained in the environment
7 through a bird-mosquito life cycle. Even if grouped with important human pathogens, USUV was
8 considered for many years as an exotic virus of minor importance confined to a small area of sub-
9 Saharan Africa and characterised by a very low pathogenicity for humans and animals.

10 In recent years, coinciding with its occurrence in Europe, the virus appears to have changed its
11 behaviour, and fatalities associated with USUV infection were reported in European wild birds
12 (Weissenböck et al., 2002; Chvala et al., 2007; Bakonyi et al., 2007; Steinmetz et al., 2010) and, in 2009,
13 two cases of human encephalitis have been associated to the USUV infection (Pecorari et al., 2009,
14 Cavrini et al., 2009). All these events have greatly changed the way of looking at these infections.
15 Information on USUV biologic features, host spectrum, and pathogenesis is becoming of high priority.
16 In Italy, before the clinical cases reported in humans, USUV was evidenced in sentinel chickens in the
17 Trentino region in 2005 (Rizzoli et al., 2007) and in the Emilia Romagna region in 2007 (Lelli et al., 2008).
18 Few fatal cases of USUV were also described in owls and blackbirds near Milan in Lombardia between
19 2006 and 2008 (Manarolla et al., 2010). As main theatre of these events, Italy definitely plays an
20 important role for this infection at both epidemiological and public health level. This paper describes
21 two years of outbreaks and USUV circulation in Italy as reported by the surveillance plan and provides
22 information to better understand the epidemiological as well as the virological characteristic of the
23 USUV Italian isolates.

24

25 MATERIALS AND METHODS

26 With the aim of monitoring flavivirus circulation in Italy, an extensive surveillance plan has been
27 implemented involving horses as well as wild and domestic birds. Insect traps were also placed in some

1 horse stables and mosquitoes were collected and identified. The surveillance activities included: i)
2 regular serological testing of sentinel-animals located in the proximity of fourteen wetland areas
3 which were selected because of the presence of migratory birds. These areas could represent a risk of
4 virus introduction and spread. The sentinel animals consisted of: horses sampled twice, in spring and in
5 late autumn, and chickens sampled as described by Monaco et al. (2008); ii) the virological
6 examination of dead wild birds and serological monitoring of backyard poultry flocks; in 2009 more
7 emphasis was placed on sampling horses, birds and mosquitoes of areas of Northern Italy in which
8 WNV and USUV have circulated in 2008 (Figure 1).

9

10 Wild birds

11 In 2009, brains, hearts, and spleens of 1045 Italian wild bird carcasses belonging to a least 22 species
12 were examined for the presence of USUV (Table 4). Between 2008 and 2009, 440 serum samples
13 belonging to 7 species were further examined for the presence of USUV antibodies.

14

15 Sentinel animals

16 According to the National surveillance plan implemented for West Nile virus since 2001, 1797 and 2567
17 chicken samples were examined serologically for USUV in 2008 and 2009, respectively. The samples
18 were from animals located in the proximity of the 14 wetland areas distributed all around Italy (Figure
19 1). Equally, from the same locations, 643 and 522 serum samples from sentinel horses were tested for
20 the presence of USUV neutralising antibodies in 2008 and 2009, respectively (Figure 1). They were
21 tested at least twice a year, before and after the mosquito season. In total, 1349 and 1275 sentinel
22 horse serum samples were examined in 2008 and 2009, respectively.

23

24 Horse sera

25 Besides the sentinel animals, additional 800 and 4130 serum samples of horses originating from all
26 Italian regions except Abruzzo and Aosta Valley, were examined for the presence of USUV antibodies
27 in 2008 and 2009, respectively.

1 Mosquito pools

2 Twenty pools of 50 mosquitoes each were tested for the presence of the USUV. They were from the
3 Tuscany region and comprised 9 pools of *C. pipiens*, 9 of *Aedes albopictus* and 2 of other species.

4

5 *Laboratory testing and diagnostic protocol*

6 Samples (blood, sera, mosquitoes and tissue) were sent for serological and virological analyses to the
7 OIE and National Reference laboratory for West Nile and Usutu viruses, Istituto G. Caporale Teramo,
8 Italy (ICT).

9 To detect the presence of USUV and WNV specific antibody response, serum samples were tested by
10 ELISA and virus neutralisation test in microtitre plates (SN) (Lelli et al., 2008). To rule out possible cross-
11 reactions, positive samples (titre $\geq 1:10$) were also tested for TBEV and WNV antibodies by PRNT (OIE,
12 2008a; Lelli et al., 2008).

13 To detect USUV in organs, EDTA blood samples and mosquitoes, a specific RT-PCR targeting the NS5
14 was used (Lelli et al., 2008; Bakonyi et al., 2007). Virus isolation was attempted in the same samples by
15 inoculating VERO E6 cell cultures. Samples negative at the first passage were sub-cultured twice in the
16 same cells (Bernard et al., 2001; Lelli et al., 2008). On blackbird carcasses collected during the
17 outbreak, bacteriological, parasitological and toxicological tests as well as virological tests other than
18 those described were also performed to exclude the presence of other pathogens. In particular,
19 samples of brain, heart, liver, kidney, spleen, lung and intestine were processed for viral isolation
20 attempts in 9-10 day old SPF chicken embryonated eggs (OIE, 2008b), chicken embryo fibroblast (CEF)
21 and chicken embryo liver (CEL). Virus isolation attempts in cells were carried out with homogenized
22 tissue samples suspended in phosphate-buffered saline with antibiotics. After centrifugation ($1,000 \times g$,
23 10 min), the supernatants were inoculated onto confluent CEF, CEL and Vero cell cultures. Cell cultures
24 were incubated at 37°C for 7 days and were checked daily for the occurrence of cytopathic effects
25 (CPE). Three blind passages were made in absence of cytopathic effect. At the end of the third
26 passage and in case of CPE, the presence of USUV in the supernatants was confirmed or excluded

1 either by electron microscopy or by Immunofluorescence (IFA) using a monoclonal antibody
2 (produced by ICT, Italy) against USUV.

3 To confirm the identification of the virus, a second USUV specific RT-PCR targeting the Pre-M region of
4 viral RNA was performed on the positive samples. The amplified products of both the Pre-M and NS5
5 were sequenced (602 bp of preM and 583 bp of NS5) and aligned with the corresponding sequences
6 deposited in Genbank (gb | AY453411.1; gb | EF206350.1) using ClustalW (table 1). NS5 datasets were
7 analyzed using the PHYLIP program (Phylogeny Interference Program Package version 3.6a)
8 (Felsenstein, 1993). For phylogenetic analysis the appropriate nucleotide substitution model selected
9 was the Kimura two-parameter model (Kimura, 1980) with transition/transversion of 2.0. Statistical
10 support at the internodes was assessed by 100 bootstrap replication.

11
12 Total RNA was extracted from samples with the High Pure Viral Nucleic Acid kit (Roche, Germany),
13 eluted in 50µl of Elution buffer according to the manufacturer instruction and used for the RT-PCR
14 reaction performed by the RT-PCR Onestep kit (Qiagen). Concisely, for each sample the mixture
15 contained 21.8 µl of Rnase free water, 10 µl of Q-solution, 2 µl of enzyme mix, 1.6 µl of each primer (50
16 pmoli/µl), 10 µl of One-step buffer 5X, 2 µl of dNTPs (10mM) and 5 µl of denatured RNA in a total
17 volume of 50 µl. The reverse transcription was performed at 45°C for 30' followed by the activation
18 phase at 95°C for 5'. Forty cycles at 95° for 30", 55°C for 30" and 72°C for 2' were used for the
19 amplification followed by 5' at 72°C for the final elongation.

20

21 *Full genome sequencing*

22 Total RNA was also extracted from 200µl of cell culture supernatant from the USUV as described below.
23 Viral RNA was reverse transcribed and amplified with a set of 66 primer pairs targeting overlapping
24 sequences of nearly 1000 bp of the viral genome. The primer sequences are available upon request.
25 Amplification and sequencing were repeated twice to avoid introduction of artificial substitutions.
26 Raw sequence data were assembled using Contig Express (Vector NTI suite 9.1, Invitrogen, USA) and
27 the consensus sequence was aligned with the Central European and South African strains (AY453411,

1 AY453412 and EF206350) to evaluate the variation within the strains. Both, the nucleotidic and the
2 deduced amino acid sequences were compared using Vector NTI suite 9.1 (Invitrogen, USA).

3

4

5 *Outbreak data*

6 Clinical signs, mortality, morbidity and macroscopic lesions observed during the outbreaks were
7 reported and fully described.

8 Serological and virological data obtained in this study were investigated and each proportion was
9 compared by using the beta distribution (Sivia, 1996).

10

11 **RESULTS**

12 *Case history*

13 Between August and October 2009 widespread anomalous death episodes of blackbirds (*Turdus*
14 *merula*) were reported in at least six different municipalities of the Treviso province (Veneto region,
15 North-Eastern Italy) (Figure 2). It was estimated that the mortality in the different outbreaks concerned
16 almost a thousand of birds altogether. From each outbreak, groups of 5-10 subjects, for a total of 35
17 blackbirds, have been examined at the Istituto Zooprofilattico Sperimentale delle Venezie and
18 comprised some dead or very ill subjects spontaneously dead few minutes after submission to the
19 laboratory.

20 At necropsy the main part of the subjects was lean and the birds showed no remarkable gross
21 changes such as hepato-splenomegaly, hepatic congestion, enlarged gallbladder, enlarged and
22 discoloured kidneys and hyperaemic meninges and brain.

23 Bacteriological and toxicological tests gave negative results. Virological tests on embryonated eggs
24 gave negative results as well. After about 3 days of incubation, focal cell rounding and shrinkage were
25 observed in VERO cell cultures inoculated with brain, heart and kidney homogenates. Within 2 days,
26 the majority of the cells showed CPEs. In detail, most of the inoculated cells lost their adherence to the

1 surface of the culturing flask and floated in the supernatant. The CPE was more evident in the
2 subsequent two passages. No CPEs or cell degeneration was observed in CEF and CEL cells.

3 The supernatants of the flasks with CPE when examined by electron microscopy showed the presence
4 of spherical viruses of about 50 nm diameter. To confirm the presence of USUV in the reported
5 outbreaks, tissue culture lysates and/or original organs from birds (no=6) representative of each
6 outbreak municipality were sent to the ICT. At the National Reference Centre USUV was then
7 detected and identified. Parasitological test highlighted the concomitant presence of
8 haemosporidian parasites in 18 of the 35 subjects examined and found positive to USUV.

9

10 *Surveillance plan*

11 Sentinel animals

12 In 2008, 9 sentinel chickens seroconverted to the USUV in the period September -October.
13 Seroconversions were observed in Tuscany, 8 in the province of Pisa and 1 in the province of Pistoia. In
14 2009, 2 sentinel chickens seroconverted in the province of Gorizia (Friuli Venezia Giulia) in the month of
15 October (Figure 2).

16 In 2008, no seroconversion was reported in horses while in 2009, 22 horses from Veneto, Emilia
17 Romagna, Tuscany and Friuli Venezia Giulia regions seroconverted (Table 2 and Figure 2).

18

19 Horses

20 When tested by SN, some sera reacted to USUV only, some others have titers for both USUV and WNV.
21 No cross reactions between the USUV and WNV were found. A higher ($P>0.05$) prevalence of positive
22 horses were found in 2008 (89.2%) compared to 2009 (7.8%). Similar results were obtained when the
23 data were analysed according to provinces and regions (Tables 3).

24

25 Wild birds

26 Virological investigation

1 One thousand and forty five tissue samples belonging to more than 22 wild bird species were tested for
2 USUV. Species and origin are listed on Table 4. Presence of USUV was detected in 11 samples, 9 (52.9%)
3 from blackbirds and 2 (0.5%) from magpies of Emilia Romagna and Veneto. Blackbird was the species
4 in which USUV was more frequently ($P>0.05$) detected (Figure 2).

5

6 Serological investigation

7 Of the 440 serum samples tested, USUV neutralising antibodies were detected in 20 rock pigeon, 1
8 blackbird and 2 magpie sera. The positive animals were from Veneto and Emilia Romagna (table 4).

9

10 *Characterisation of the isolates*

11 Sequence comparison

12 Results of the alignment of NS5 are shown in figure 3. No differences were observed among the Italian
13 USUV strains isolated from Veneto, Friuli and Emilia Romagna regions when the NS5 partial sequences
14 were aligned. Sequences were identical to those of the strain detected in the brain of the human
15 patient and shared a high similarity with those of the Vienna, Budapest and PI-09-Cp isolates. The NS5
16 partial sequence of the Italian strain detected in *C. pipiens* caught in Tuscany was more similar to
17 Budapest and Vienna isolates than to the other Italian isolates.

18 High degree of similarity at both nucleotide and aminoacid level was also found when the full genome
19 sequence of the Italian isolate (RA-09-Tm) was compared with that of the strains circulating in Europe,
20 (Table 5). The Italian USUV strain sequences exhibited 97% identity to the South African reference strain
21 SAAR-1776. The nucleotide sequences were translated to putative amino acid sequences. The
22 deduced amino acid sequences of the Italian strain differed by 10 and 11 amino-acids from the
23 Budapest and Vienna strains, respectively (Table 6). The Italian USUV showed 99% amino acid identity
24 to the SAAR-1776 strain. The amino acid differences are summarized in table 6.

25

26 **DISCUSSION**

1 In the last decades the emergence of new diseases and/or the recrudescence of old disease into new
2 areas exhorted the scientific community to propose a new category of pathogens: the so called
3 emerging and re-emerging pathogens. Among the emerging pathogens, arboviruses, which by
4 definition are viruses that are transmitted to vertebrates by arthropods (mosquitoes, ticks, sandflies,
5 midges ...), are nowadays definitely playing an important role. USUV is an arbovirus and, in view of the
6 recent events, it represents the typical example of an emerging pathogen. It appeared in Austria in
7 2001 causing widespread mortality among the population of Eurasian blackbirds and some other bird
8 species (Weissenböck et al., 2002). In the following years presence of USUV was associated to mortality
9 of birds south east (Hungary) and west (Switzerland) of Austria (Bakonyi et al., 2007; Steinmetz et al.,
10 2010). In Italy a silent virus circulation was detected since 2007 (Lelli et al., 2008). In the last years
11 presence of this virus has been associated with fatal cases in birds. More recently the Italian strain was
12 detected in the brain of immune deficient patients with neuroinvasive infection (Pecorari et al., 2009;
13 Cavrini et al., 2009). The data of this study prove that, after being evidenced in 2007, USUV has
14 circulated widely in Italy at least in Tuscany, Veneto, Emilia Romagna and Friuli regions.
15 Seroconversions of either sentinel horses or chickens in both 2008 and 2009 and the detection of virus in
16 wild birds, support the hypothesis of an endemic cycle in local bird and mosquito species able to
17 maintain and disperse USUV in Italy. A rather complex relationship between virus, hosts and vectors is
18 needed to maintain an arbovirus in the environment. Vector competence for a particular virus and
19 the contemporary presence of susceptible vertebrate host species with viraemia levels permitting
20 vector transmission, are key factors for the spread of the virus in a given environment. USUV is thought
21 to be maintained in nature by a mosquito-bird transmission cycle. In Africa ornithophilic mosquitoes of
22 the genera *Culex*, *Coquillettidia* and *Mansonia* are thought to be the main vectors (Pfeffer and
23 Dobler, 2010). In Austria *Culex* spp may play a major role (Pfeffer and Dobler, 2010), although USUV has
24 not been isolated from mosquitoes so far but it has been only detected in overwintering *Culex pipiens*
25 pools by real time RT-PCR. In this study USUV was detected in a pool of *C. pipiens* and was isolated
26 from organs of dead blackbirds suggesting that blackbirds and *C. pipiens* might play an important role
27 in the Italian endemic cycle. The fact that many horses often had neutralising titers to both WNV and

1 USUV seems to imply that both viruses might share the same vector/s. It is now well documented that
2 *C. pipiens* represents the main vector of WNV in Italy and its involvement as a vector has also been
3 suggested for USUV (Tamba et al., 2010). *Culex pipiens* is commonly regarded as ornithophilic (Hamers
4 et al., 2008; Marra et al., 2004; Turell et al., 2005; Simpson et al., 2009). In the United States of America it
5 has been observed that it displayed an innate host preference for *Turdus* genus (Simpson et al., 2009).
6 Preferential feeding by a vector on virus-competent hosts may influence transmission dynamics as
7 contact rates are shifted away from other abundant, but less competent hosts (Simpson et al., 2009). In
8 USUV infection, the blackbird (*Turdus merula*), a species of the *Turdus* genus, has been the one most
9 commonly involved. Other studies showed that also certain owl species and house sparrows are
10 susceptible to USUV infection (Manarolla et al., 2009; Steinmetz et al., 2010). A considerable number of
11 different bird species were examined in this study, USUV however was detected in magpies and
12 blackbirds only. As in Central Europe (Chvala et al., 2007; Bakonyi et al., 2007; Steinmetz et al., 2010),
13 the blackbird was the species found more significantly affected (81.9%). In this species as reported in
14 the province of Treviso, USUV was able to determine clinical signs and high mortality rate with
15 symptoms and lesions resembling those observed in Austria (Weissenböck et al., 2002; Chvala et al.,
16 2004).

17 USUV has been detected in 9 out of 11 carcasses of birds which died because of nervous symptoms.

18 **Six of them were from the outbreaks reported in the province of Treviso.**

19 Detection of USUV in dead birds does not prove that it is the cause of the death of the animal. Excess
20 deaths occurring in a restricted area and the detection of USUV in all dead birds tested as occurred in
21 the Treviso area might indicate that the bird deaths, are likely to be due to USUV. **Almost 50% of the**
22 blackbirds examined in this study were also parasitised by hemosporidia. Presence of parasites in free-
23 ranging wild birds are a common finding (Cole and Friend, 1999). They debilitate the hosts, and thus
24 might facilitate USUV infections. Although other species like pigeons and magpies have been found
25 serologically and/or virologically infected, no reports of mass die-offs of species other than blackbirds
26 were recorded. This indicates that several species of birds are susceptible to USUV infection, but only a
27 few get fatal disease. In view of the fact that Usutu virus infects wild birds and causes high death rates,

1 especially in blackbirds, the finding that the chicken embryos, CEF, CEL were apparently resistant to
2 Usutu virus infection could appear unexpected. Other authors, however, have found a similar result
3 when infecting chicken embryo and CEF with USUV (Bakonyi et al., 2005).

4 Dramatic bird mortality could be due to a particular feeding preference of the vector, to a particular
5 susceptibility of the host species, being completely naive in the new endemic areas or, alternatively, to
6 the high pathogenicity of some USUV strains for certain bird species because of genetic mutations. Like
7 the Central European strains, the Italian North Eastern strains were pathogenic and capable of causing
8 mortality in wild birds, particularly in blackbirds. The Italian North eastern strains however were also able
9 to infect humans and cause encephalitis. Considering that *C. pipiens*, the likely vector of USUV, has
10 been demonstrated to serve both as an enzootic and a bridge vector for humans (Hamer et al.,
11 2008), USUV infection in humans was not surprising. What was surprising, was the encephalitis
12 associated to the infection.

13 It has been shown that neurovirulence and neuroinvasiveness of Flaviviruses are mainly associated with
14 sequence variation of the protein E and to a lesser extent to other viral proteins such as NS1 and NS2b
15 (Beasley et al., 2005; Brault et al., 2004; Botha et al., 2008). The RA-09-Tm, a strain isolated from a
16 blackbird in the same area where the human infection occurred, was fully sequenced and sequences
17 compared with those of the Budapest, Vienna and South African isolates. The full genome sequence
18 of the USUV isolated in North Eastern Italy grouped with the Budapest and Vienna isolates sharing 99%
19 of similarity differing from both European isolates in 10-11 amino-acids only. These amino-acid
20 substitutions were distributed throughout the entire genome. No differences were noticed in the
21 amino-acid sequences of the protein E, the most commonly referred as potential site of pathogen
22 determinants. It seems then that other host related variables such as susceptibility, age, immune and
23 general health status might have affected the USUV pathogenicity for humans.

24 The genetic analysis also confirmed that as a whole the European USUV isolate cluster is closely related
25 to the South African USUV. Their nucleotidic and amino acidic sequences shared 97% and 99%
26 similarity, respectively. The minor differences observed in the amino acidic sequence were the same
27 reported by Bakonyi et al. (2004). These authors, assumed that the Vienna strain evolved

1 independently from the South African USUV. A different picture however emerged when the NS5
2 partial sequences of the Italian, South African and Central European isolates were compared. Three
3 genetically different clusters could be evidenced. One included all strains isolated in the North Eastern
4 regions of Italy, a second which grouped the Central European and the Tuscany strains and a third
5 which comprised the South African strain. The NS5 583 bp partial sequences of the North Eastern and
6 Tuscany isolates differ in 5 nucleotides. With all caution induced by the limited NS5 fragment
7 sequenced, one might also hypothesize that not all the Italian strains clustered together. The Tuscany
8 strain might indeed differ from the other Italian isolates, being closer to the Central European isolates
9 and for it a further different origin could be hypothesised. According to the seroconversion observed
10 either in horses or in chicken, USUV has circulated in Tuscany during 2008 and 2009. Unlike the other
11 Italian USUV strains, the Tuscany strain circulation as shown by the horse and chicken seroconversions
12 did not coincide with excess bird mortality. In the same area a massive blackbird mortality was
13 however described in 1996 (Mani et al., 1998). At that time, a JEV-like virus (Mani et al., 2009) was
14 identified as the virus responsible of the outbreak. It could be supposed that the Tuscany USUV strain
15 might have been circulating in Italy at least since 1996 and might be the cause of excess mortality in
16 naive bird population. It might be further hypothesised that it might have established subsequently an
17 efficient silent transmission cycle between local bird and mosquito species. It has been shown that
18 after an initial severe bird mortality, the virus might become endemic. The high seroprevalence
19 observed in these situations in the susceptible hosts might be an indication of the establishment of a
20 stable population immunity which, however, does not preclude virus activity with new infections and
21 seroconversions without causing further excess mortality (Meister et al., 2007). The seroconversion in
22 sentinel animals in both 2008 and 2009 implied the presence of active virus circulation in Tuscany. In
23 accordance with the horse seroprevalence data, the virus activity in this region (31.58%) was probably
24 much lower than that occurring in the same year in the North Eastern regions (>90%). In 2008
25 incidence of USUV in horses in the North eastern regions was high and decreased markedly in the
26 following year. In birds it has been observed that even after natural infection flaviviral antibody titres
27 are generally low and subject to considerable variations within short times (Meister et al., 2007). A

1 similar phenomenon could have occurred in the horse population tested. In this species USUV
2 infection generally does not induce high antibody titers. Horses are not susceptible to Usutu and do
3 not show a strong immune response, however they appear to be good sentinel animals to monitor
4 USUV circulation.

5

6 **CONCLUSIONS**

7 This study suggests the possibility that two strains of USUVs have circulated in Italy between 2008 and
8 2009. They have developed strategies of adaptation and evolution to spread into new areas and
9 become established. Their circulation has been associated to high blackbird mortality and, although
10 sharing 100% similarity at amino acidic level with the other European strains, at least the Italian North
11 eastern strain showed a distinctive zoonotic potential. Keeping in mind that dealing with a member of
12 the genus *Flavivirus* the unexpected is often the most frequent event particularly with respect to
13 disease severity, unusual clinical manifestations, and unpredicted methods of transmission, further
14 investigations involving different bird and mammal species are still necessary to show the most
15 important host species, natural reservoirs, and vectors of Usutu virus and to estimate its epidemiologic
16 impact and possible threat to domesticated animals and to the human population. This information will
17 allow us to better understand the diffusion routes and dynamics of potential zoonotic arboviruses
18 and know better the different elements of their complex biological cycle. Therefore it will be possible to
19 design more efficient surveillance, control and prevention activities for these emerging viral zoonoses
20 with great benefit for human and veterinary public health.

21

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Table 1

Strains of Usutu viruses included in NS5 and PrM partial sequencing

| Date | ID | Bankit/GenBank | Biological matrix | Species | Geographical Origin | NS5 | PrM |
|-------------|-------------|-----------------------|--------------------------|------------------------------|----------------------------|------------|------------|
| 01/09/09 | TV1-09-Tm | 1430426 | organs | <i>Blackbird (T. merula)</i> | Treviso (Veneto) | x | x |
| 16/09/09 | TV2-09-Tm | 1430425 | organs | <i>Blackbird (T. merula)</i> | Treviso (Veneto) | x | |
| 18/09/09 | UD-09-Tm | 1430431 | organs | <i>Blackbird (T. merula)</i> | Udine (Friuli) | x | |
| 23/10/09 | MO-09-Hu | 1430429 | cerebral spinal fluid | <i>Human</i> | Modena (Emilia Rom.) | x | x |
| 20/10/09 | RA-09-Tm | 1430432 | organs | <i>Blackbird (T. merula)</i> | Ravenna (Emilia Rom.) | x | x |
| 29/09/09 | TV3-09-Tm | 1430433 | organs | <i>Blackbird (T. merula)</i> | Treviso (Veneto) | x | x |
| 07/10/09 | VE-09-Tm | 1430428 | organs | <i>Blackbird (T. merula)</i> | Venezia (Veneto) | x | |
| 2009 | PI-09-Cp | 1430405 | mosquitoes | <i>Culex pipiens</i> | Tuscany | x | |
| 2001 | Vienna'01 | AY453411 | organs | <i>Blackbird (T. merula)</i> | Austria | x | |
| 1959 | SAAR-1776 | AY453412 | mosquitoes | <i>Culex neavei</i> | South Africa | x | |
| 2005 | Budapest'05 | EF206350 | organs | <i>Blackbird (T. merula)</i> | Hungary | x | |

Table 2

Seroconversions to Usutu virus in horses in 2009

| Region | Province | Number of seroconversions |
|-----------------------|---------------|---------------------------|
| EMILIA ROMAGNA | FERRARA | 5 |
| | | 1 |
| | REGGIO EMILIA | 5 |
| FRIULI VENEZIA GIULIA | UDINE | 1 |
| TUSCANY | FIRENZE | 1 |
| | LUCCA | 1 |
| | PISA | 1 |
| | PISTOIA | 4 |
| VENETO | VERONA | 2 |
| | VICENZA | 1 |

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Table 3

Usutu virus seroprevalence in Italian horses in 2008-2009 according to regions

| Year | Region | No. of horses tested | No. of positives | % of positives |
|---------------------|-----------------------|----------------------|------------------|----------------|
| 2008 | CAMPANIA | 2 | 0 | 0,00 |
| | EMILIA ROMAGNA | 371 | 366 | 98,65 |
| | FRIULI VENEZIA GIULIA | 4 | 1 | 25,00 |
| | LAZIO | 13 | 0 | 0,00 |
| | LOMBARDY | 112 | 109 | 97,32 |
| | MARCHE | 12 | 0 | 0,00 |
| | SICILY | 12 | 0 | 0,00 |
| | TUSCANY | 19 | 6 | 31,58 |
| | VENETO | 255 | 227 | 89,02 |
| | Total | 800 | 709 | 88,6 |
| 2009 | BASILICATA | 207 | 0 | 0,00 |
| | CALABRIA | 71 | 0 | 0,00 |
| | CAMPANIA | 84 | 0 | 0,00 |
| | EMILIA ROMAGNA | 739 | 100 | 13,53 |
| | FRIULI VENEZIA GIULIA | 207 | 6 | 2,90 |
| | LAZIO | 619 | 0 | 0,00 |
| | LIGURIA | 3 | 0 | 0,00 |
| | LOMBARDY | 195 | 20 | 10,26 |
| | MARCHE | 162 | 0 | 0,00 |
| | MOLISE | 96 | 0 | 0,00 |
| | PIEDMONT | 1 | 0 | 0,00 |
| | APULIA | 59 | 0 | 0,00 |
| | SARDINIA | 86 | 0 | 0,00 |
| | SICILY | 280 | 0 | 0,00 |
| | TUSCANY | 404 | 22 | 5,45 |
| | TRENTINO ALTO ADIGE | 1 | 0 | 0,00 |
| | UMBRIA | 65 | 0 | 0,00 |
| | VENETO | 851 | 203 | 23,85 |
| Total | 4130 | 351 | 8,5 | |
| Total 2008 and 2009 | | 4930 | 1060 | 21,5 |

Table 4

Virological and serological findings in Italian wild birds examined for USUTU virus in the years 2008-2009

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| Region | Species | Common name | RT-PCR | | Serum-neutralisation | |
|---------------------------------|------------------------------------|-----------------------------|-----------------------|------------------|-----------------------|------------------|
| | | | No. of animals tested | No. of positives | No. of animals tested | No. of positives |
| BASILICATA | <i>Columba livia</i> | Rock pigeon | 1 | 0 | 0 | 0 |
| | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 2 | 0 |
| CAMPANIA | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 1 | 0 |
| | - | Wild birds | 1 | 0 | 0 | 0 |
| EMILIA ROMAGNA | <i>Otus scops</i> | Eurasian scops owl | 1 | 0 | 0 | 0 |
| | <i>Accipiter gentilis</i> | Goshawk | 1 | 0 | 0 | 0 |
| | <i>Tyto alba</i> | Barn owl | 1 | 0 | 0 | 0 |
| | <i>Athena noctua</i> | Little owl | 2 | 0 | 0 | 0 |
| | <i>Columba livia</i> | Rock pigeon | 1 | 0 | 17 | 17 |
| | <i>Corvus corone corvix</i> | Carrion crow | 147 | 0 | 0 | 0 |
| | <i>Corvus corax</i> | Common raven | 2 | 0 | 0 | 0 |
| | <i>Larus occidentalis</i> | Seagull | 8 | 0 | 0 | 0 |
| | <i>Pica pica</i> | Magpie | 223 | 2 | 0 | 0 |
| | <i>Anas platyrhynchos</i> | Mallard | 1 | 0 | 0 | 0 |
| | <i>Garrulus glandarius</i> | European jay | 21 | 0 | 0 | 0 |
| | <i>Bubo bubus</i> | Eurasian eagle owl | 1 | 0 | 0 | 0 |
| | <i>Turdus merula</i> | Blackbird | 8 | 2 | 0 | 0 |
| | <i>Erithacus rubecula</i> | European robin | 1 | 0 | 0 | 0 |
| | <i>Columba livia</i> | Rock pigeon | 1 | 0 | 0 | 0 |
| | <i>Accipiter nisus</i> | Eurasian sparrow | 2 | 0 | 0 | 0 |
| | <i>Sturnus vulgaris</i> | Common starling | 112 | 0 | 0 | 0 |
| | <i>Streptopelia turtur</i> | Eurasian collared dove | 1 | 0 | 0 | 0 |
| | FRIULI VENEZIA GIULIA | <i>Corvus corone corvix</i> | Carrion crow | 3 | 0 | 6 |
| <i>Corvus corax</i> | | Common raven | 0 | 0 | 1 | 0 |
| <i>Pica pica</i> | | Magpie | 39 | 0 | 80 | 0 |
| <i>Gallus gallus domesticus</i> | | Chicken | 0 | 0 | 5 | 2 |
| LAZIO | <i>Anas platyrhynchos</i> | Mallard | 0 | 0 | 1 | 0 |
| | <i>Corvus corone corvix</i> | Carrion crow | 38 | 0 | 0 | 0 |
| MARCHE | <i>Corvus corax</i> | Common raven | 5 | 0 | 0 | 0 |
| | <i>Pica pica</i> | Magpie | 20 | 0 | 0 | 0 |
| | <i>Sturnus vulgaris</i> | Common starling | 1 | 0 | 0 | 0 |
| MOLISE | <i>Gallus gallus domesticus</i> | Chicken | 2 | 0 | 8 | 0 |
| SARDINIA | <i>Corvus corone corvix</i> | Carrion crow | 8 | 0 | 0 | 0 |
| | <i>Phoenicopterus ruber roseus</i> | Greater flamingo | 0 | 0 | 1 | 0 |
| | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 3 | 0 |
| SICILY | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 14 | 0 |
| TUSCANY | <i>Anas platyrhynchos</i> | Mallard | 0 | 0 | 6 | 6 |
| | <i>Columba livia</i> | Rock pigeon | 159 | 0 | 0 | 0 |
| | <i>Pica pica</i> | Magpie | 4 | 0 | 0 | 0 |
| | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 5 | 5 |
| | <i>Turdus philomelos</i> | Song thrush | 7 | 0 | 0 | 0 |
| | <i>Streptopelia turtur</i> | Eurasian collared dove | 12 | 0 | 0 | 0 |
| TRENTINO | <i>Tetrao urogallus</i> | Capercaille | 1 | 0 | 0 | 0 |
| UMBRIA | <i>Corvus corax</i> | Common raven | 0 | 0 | 73 | 0 |
| | <i>Pica pica</i> | Magpie | 0 | 0 | 57 | 0 |
| | <i>Gallus gallus domesticus</i> | Chicken | 0 | 0 | 1 | 0 |
| VENETO | <i>Columba palumbus</i> | Wood pigeon | 0 | 0 | 1 | 0 |
| | <i>Columba livia</i> | Rock pigeon | 0 | 0 | 135 | 3 |
| | <i>Corvus corone corvix</i> | Carrion crow | 55 | 0 | 3 | 0 |
| | <i>Fringilla coelebs</i> | Chaffinch | 1 | 0 | 0 | 0 |
| | <i>Larus occidentalis</i> | Seagull | 6 | 0 | 0 | 0 |
| | <i>Pica pica</i> | Magpie | 113 | 0 | 17 | 2 |
| | <i>Turdus merula</i> | Blackbird | 9 | 7 | 1 | 1 |
| | <i>Erithacus rubecula</i> | European robin | 1 | 0 | 0 | 0 |
| | <i>Sturnus vulgaris</i> | Common starling | 3 | 0 | 0 | 0 |
| | <i>Turdus philomelos</i> | Song thrush | 22 | 0 | 0 | 0 |
| | <i>Streptopelia turtur</i> | Eurasian collared dove | 6 | 0 | 0 | 0 |
| | Wild birds | 5 | 0 | 0 | 0 | |
| Total | | | 1045 | 11 | 404 | 36 |

Table 5

Percentage of nucleotidic and amino-acid sequence similarity between European and South African USUTU strains

Legend:

Vienna'01: Vienna strain 2001

SAAR-1776: South African strain SAAR-1776

Budapest'05: Budapest strain 05

RA-09-Tm: Italian strain Ravenna 2009

| | Vienna'01 | SAAR-1776 | Budapest'05 | RA-09-Tm |
|-------------|-----------|-----------|-------------|----------|
| Vienna'01 | | 97 | 100 | 99 |
| SAAR-1776 | 99 | | 97 | 97 |
| Budapest'05 | 100 | 99 | | 99 |
| Italy'09 | 100 | 99 | 100 | |

aa

Table 6

Comparison between the amino-acid deduced sequences of the European and South African Usutu strains

| Position bp | Vienna'01 | SAAR-1776 | Budapest'05 | RA-09-Tm | Position aa | Gene | Vienna'01 | SAAR-1776 | Budapest'05 | RA-09-Tm |
|-------------|-----------|-----------|-------------|----------|-------------|------|-----------|-----------|-------------|----------|
| 454-56 | GTT | GCC | GTT | GTT | 120 | C | val | ala | val | val |
| 622-24 | ACT | ACT | ATT | ACT | 176 | M | thr | thr | ila | thr |
| 637-39 | AAA | AAA | AAA | ACT | 181 | | lys | lys | lys | arg |
| 1351-53 | ATG | GTG | ATG | ATG | 419 | E | met | val | met | met |
| 1801-03 | GGC | AGC | GGC | GGC | 569 | | gly | ser | gly | gly |
| 1933-35 | GGC | AGC | GGC | GGC | 613 | | gly | ser | gly | gly |
| 2242-44 | ACA | CCA | ACA | ACA | 716 | | thr | pro | thr | thr |
| 2464-66 | AAC | AGC | AAC | AAC | 790 | | asn | ser | asn | asn |
| 2584-86 | GAA | GAG | GAA | GGA | 830 | NS1 | glu | glu | glu | gly |
| 2911-13 | GCA | GTA | GCA | GTA | 939 | | asn | val | asn | val |
| 3445-47 | AGG | AAG | AGG | AGG | 1117 | | arg | lys | arg | arg |
| 3532-34 | CAT | TAC | CAT | TAT | 1146 | NS2a | his | tyr | his | tyr |
| 3685-87 | ACT | ACT | ATT | ACT | 1197 | | thr | thr | ile | thr |
| 3895-97 | AAC | GAC | AAC | AAC | 1267 | | asn | asp | asn | asn |
| 3898-900 | TTT | CTC | TTT | TTT | 1268 | | phe | leu | phe | phe |
| 4066-68 | ATC | ATT | ACC | ACC | 1324 | | ile | ile | thr | thr |
| 4900-02 | ATA | ATA | ATA | GTA | 1602 | NS3 | ile | ile | ile | val |
| 4948-50 | ATC | GTC | ATC | ATC | 1618 | | ile | val | ile | ile |
| 5179-81 | CTG | ATG | CTG | CTG | 1695 | | leu | met | leu | leu |
| 5431-33 | GCT | GTT | GTT | GTT | 1779 | | ala | val | val | val |
| 6184-86 | AGA | ATA | AGA | AGA | 2030 | | arg | ile | arg | arg |
| 6190-02 | GAG | CAG | GAG | GAG | 2032 | | glu | gln | glu | glu |
| 6412-14 | GCT | TCC | GCT | GCT | 2106 | | ala | ser | ala | ala |
| 6592-94 | CTT | TTT | CTT | CTT | 2166 | NS4a | leu | phe | leu | leu |
| 6955-57 | ATA | ATG | ATA | ATA | 2287 | NS4b | ile | met | ile | ile |
| 6964-66 | AGC | GGC | AGC | AGC | 2290 | | ser | gly | ser | ser |
| 7006-08 | AAC | AAC | AAC | AGC | 2304 | | asn | asn | asn | ser |
| 7195-97 | TTA | TTG | TTG | CTG | 2367 | | phe | leu | leu | leu |
| 7744-46 | AAG | AGG | AAG | AAG | 2550 | NS5 | lys | arg | lys | lys |
| 8029-31 | ATG | ATG | ATG | ATA | 2645 | | met | met | met | ile |
| 8503-05 | ACC | GCC | ACC | ACC | 2803 | | thr | ala | thr | thr |
| 8641-43 | AGC | GGC | AGC | AGC | 2849 | | ser | gly | ser | ser |
| 10183-85 | CCA | CCA | CCA | CTA | 3363 | | pro | pro | pro | leu |
| 10375-77 | AAT | GAT | AAT | AAT | 3427 | | asn | asp | asn | asn |

Figure 1

Areas surveyed for the presence of Usutu virus and Usutu virus antibodies in 2008 and 2009

Figure 2

Usutu virus circulation in Italy in 2008 and 2009

Figure 3

Neighbor joining tree compiled from aligned nucleic acid sequences of the partial NS5 segments of Usutu virus isolates. The tree is rooted with the same sequence of the Eg101 WNV strain. The most significant support value from the bootstrap analysis is reported.

Legend:

Eg101: West Nile strain Egypt 101

PI 09 Cp: Usutu strain detected in *C. pipiens* from Tuscany in 2009

Vienna01: Usutu strain Vienna 2001

Budapest05: Usutu strain Budapest 2005

SAAR-1776: Usutu strain from South African 1959

VE 09 Tm: Usutu strain isolated in 2009 in the province of Venice from blackbird (*T. merula*)

TV1 09 Tm: Usutu strain isolated in 2009 in the province of Treviso from blackbird (*T. merula*)

TV2 09 Tm: Usutu strain isolated in 2009 in the province of Treviso from blackbird (*T. merula*)

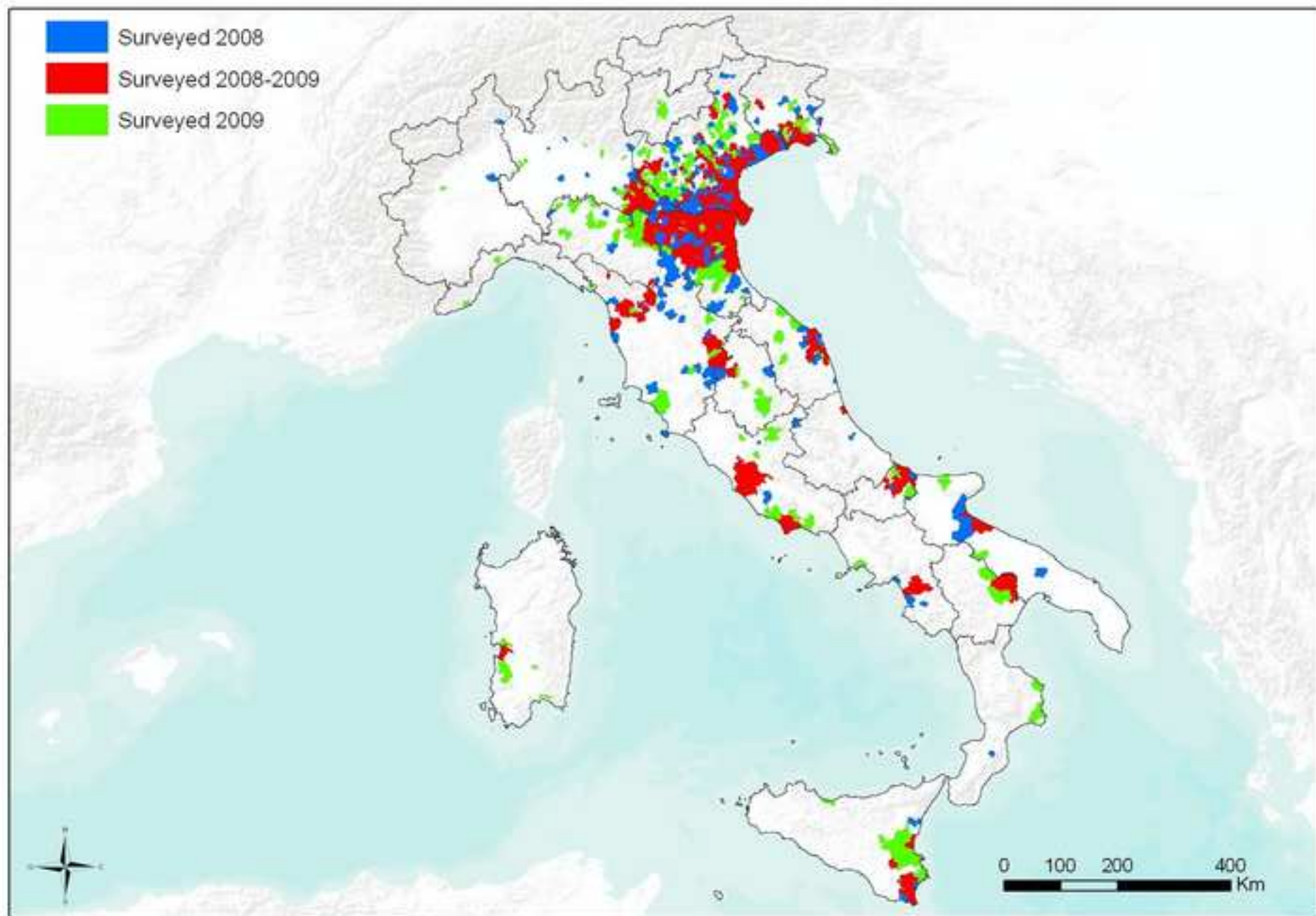
TV3 09 Tm: Usutu strain isolated in 2009 in the province of Treviso from blackbird (*T. merula*)

MO 09 Tm: Usutu strain isolated in 2009 in the province of Modena from blackbird (*T. merula*)

RA 09 Tm: Usutu strain isolated in 2009 in the province of Ravenna from blackbird (*T. merula*)

UD 09 Tm: Usutu strain isolated in 2009 in the province of Udine from blackbird (*T. merula*)

crip



crip

