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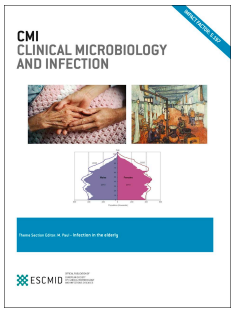

Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy

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ABSTRACT

Objectives: We aimed to monitor the spread and to evaluate the role for public health of Usutu virus (USUV) in an endemic area of Italy.

Methods: The survey was retrospectively conducted by detecting USUV-RNA and USUV-antibodies in cerebrospinal fluid (CSF) and serum samples collected between 2008 and 2011 from 915 patients with or without neurological impairments in the area of the municipality of Modena, Italy. Real time RT-PCR was performed on CSF and serum specimens while serum-neutralization was used to detect antibodies. Organs of birds and pools of mosquitoes were also tested for USUV RNA. Positive samples were partially sequenced and used for phylogenetic analysis.

Results: The presence of USUV RNA (1.1%; CI 95%: 0.6-2.0%) was significantly ($p < 0.05$) higher than that of West Nile virus (WNV) (0%; CI 95%: 0%-0.33%). USUV antibodies level was 6.57% (CI 95%: 4.87-8.82%) and it was significantly higher ($p < 0.05$) compared to that of WNV (P= 2.96%, CI 95%: 1.89-4.62%). Partial genome sequencing of USUV strains detected in humans, birds and mosquitoes revealed high nucleotide sequence identity within them and with the USUV strains isolated in Central Europe.

Conclusions: USUV infection in humans is not a sporadic event in the studied area and the USUV neuro-invasiveness has been confirmed.

INTRODUCTION

As confirmed by the recent Zika virus outbreak in humans, there is compelling evidence that the epidemiological scenario of infectious diseases is unstable depending on climatic, ecological and human-related factors. Possible consequences are the emergence and spread of new pathogens in new areas as well as the re-emergence of well-known or neglected pathogens with altered virulence and transmission features or different host/tissue tropism [1, 2]. Within this milieu, Usutu virus (USUV) may be considered as an emerging pathogen. USUV is an arthropod-borne flavivirus that belongs to the Japanese encephalitis serocomplex together with West Nile virus (WNV). Its natural life cycle involves ornithophilic mosquitoes as vectors (mainly *Culex* spp) and birds, mainly wild birds, as amplifying hosts. Mammalian including horses, dogs, wild boars and humans may act as accidental hosts [3-5]. As USUV transmission requires competent vectors, in temperate climate zones virus circulation is limited to some periods of the year, usually late spring or early autumn [3]. Among vertebrate hosts, migratory birds play a pivotal role in spreading USUV into new areas [4, 6]. Originally isolated in the Republic of South Africa in 1958 from *Culex navei*, USUV was not considered for long time as a threat for humans and animals [3, 7]. Only two USUV related infections had officially been reported in humans in Africa including a man with fever and skin rash (Central African Republic, 1981) and a ten year old child with fever and jaundice [3]. In the last twenty years, however, the epidemiological scenario of USUV changed considerably. Since 1996, USUV has been demonstrated to be responsible for several outbreaks of overt disease in birds in Southern and Central-Eastern Europe [8-12]. Specifically, USUV infections have been observed to be responsible for severe neurological symptoms, often fatal, in wild and domestic birds. In 2009, two human cases of severe encephalitis due to USUV infection were reported in Italy, at the Azienda Ospedaliero-Universitaria Policlinico (AOUP) of Modena [13, 14], and additional USUV neuroinvasive infections in humans were described in Croatia [15, 16] and in a retrospective study in Italy [17].

As for the continuous incursions of WNV through migratory birds, the Italian Ministry of Health has implemented since 2001 a National Surveillance plan for this virus. Starting from 2003, diagnostic activities dedicated to USUV have also been regularly performed on samples coming from sentinel animals (horses and chickens), mosquitoes and birds as integration of the WNV plan [4].

These engagements gave to the Italian Health authorities the possibility to monitor over the years the circulation of USUV in animals and insects and to realize that, analogously to WNV, also this virus has become a resident pathogen. This epidemiological scenario in conjunction with the increased zoonotic potential of this virus caused great public health concern [4]. Thus, the evaluation of the role of USUV on public health has become crucial and further investigations were therefore required. In this perspective, the presence of USUV RNA and USUV-specific antibodies was retrospectively investigated by molecular and serological assays on cerebrospinal fluid and serum samples collected from patients admitted or tested at the AOUP of Modena between 2008 and 2011. The viral genomes detected were partially sequenced and compared with homologous sequences of USUV strains detected in human, animal or vector samples over the years.

MATERIALS AND METHODS

Human samples

At the end of 2012 a total of 915 human specimens were retrospectively selected at the AOUP of Modena (Emilia-Romagna region)-Italy. Out of 915 samples, 306 were cerebrospinal fluid (CSF) samples collected between 2008 and 2009 from patients with suspected viral encephalitis or meningoencephalitis which resulted negative for neurotropic viruses (Cytomegalovirus, Herpes simplex virus type 1 and type 2, Epstein–Barr virus, Adenovirus, Varicella-Zoster virus, Human herpesvirus 6, Hepatitis E virus) previously tested by molecular methods, whereas 609 were serum samples collected between 2008 and 2011 from inpatients/outpatients including healthy (pregnancy screening and for pre- and post-vaccinal control) and sick subjects (patients with oncological, respiratory and infectious diseases) and tested for serological markers of viral infections. CSF and serum samples were HIV-negative and they had not been tested for *Flavivirus* genus. CSF and serum samples were selected within those collected in the period of presence of

USUV and WNV vectors (mosquitoes) in our country (from June to November of each year) and within those with a suitable amount of material for molecular and serological analyses. Haemolysed serum samples were discarded. All selected samples were then simultaneously analysed; in particular CSFs were tested for the presence of USUV and WNV RNAs while serum samples were examined for the presence of USUV or WNV RNAs and neutralising antibodies for both viruses. This project was approved by the Modena's Provincial Ethics Committee and registered with protocol no. 2797, 11/07/2012.

Resident birds and mosquito pools

Spleen, brain, heart tissue samples, collected between 2010 and 2015 during the National WNV surveillance plan, from several species of dead birds were also tested for the presence of USUV RNA as well as pools of *Culex pipiens* captured in several Italian regions.

Molecular methods

RNA purification

Total RNA purification from CSFs, serum, tissue and mosquito samples was carried out using the High Pure Viral Nucleic Acid kit (Roche, Basel CH) according to the manufacturer's instruction.

One-Step RT-PCR for USUV

Purified RNA was then tested by using a previously described RT-PCR (RT_{USUV}) assay [9] which targets a 450 bp fragment of NS5 gene encoding for the viral polymerase. RT_{USUV} reaction was achieved with the QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden DE) according to the manufacturer's instruction and the amplification was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) with the following thermal profile: 30 min of reverse transcription at 45°C, 5 min of polymerase activation at 95°C and 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s followed by a final extension of 5 min at 72°C.

One-Step RT-PCR Real-time for WNV

Samples were also tested for WNV RNA. A quantitative real-time RT-PCR ($qPCR_{wnv}$, [18]) assay was performed with the SuperScript™ III RT/Platinum one-step quantitative RT-PCR system kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). $qPCR_{WNV}$ was carried out in the 7900HT Fast Real-Time PCR

Systems (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) under the following conditions: 50°C for 15 min, 95°C for 2 min and 40 cycles at 95°C for 15 sec and 60°C for 30 sec.

Sequencing

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden DE) and sequenced by BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The sequence reactions were analyzed on 3130 XL Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA); raw sequence data were assembled using Contig Express (Vector NTI suite 9.1, Invitrogen, Thermo Fisher Scientific, Waltham, MA) and consensus sequences were compared to public databases by blast algorithm to exclude sequencing artefacts (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The phylogenetic analysis was carried out on a dataset of NS5 gene fragment nucleotide sequences employing all USUV sequences publicly available. Nucleotide sequences were aligned with MAFFT v7 [19] and manually refined. Maximum-Likelihood tree (Fig 1) was estimated with MEGA6 [20] using the K2+I (Best DNA model) as substitution model. Branch support was assessed with 500 bootstrap replicates. The option “Display only topology” has been used for graphical representation of the analysis.

Serum-neutralization

Serum samples were also examined for the presence of neutralising antibodies by serum-neutralization for USUV and WNV according to the method recently described by the OIE Reference Laboratory for West Nile Fever of Teramo [21].

RESULTS

Molecular data

Human samples

USUV RNA was detected in 8 CSFs (P= 2.61%; CI 95%: 1.35%-5.07%), 7 CSFs collected in 2008 and one in 2009, and in 2 serum samples collected in 2009 (P= 0.33%; CI 95%: 0.10%-1.18%) (Table 1A). All samples turned out negative when tested by $qPCR_{wnv}$ (Table 1B). Positive samples originated from four male individuals with average (standard deviation) age of 49.75 (16.58) while the remaining originated from four

female individuals, 51.50 (12.81). Anamnestic and clinical data were available only for 4/8 cases with USUV neuroinvasive infection (Table 2). Briefly, two patients were admitted with meningoencephalitis, one with acute encephalitis and one with acute encephalitis and polyneuritis. All of them had an underlying chronic disease (liver disease, chronic obstructive pulmonary disease/diabetes, hypertension and aortic/mitral insufficiency, respectively).

Unfortunately we were not able to provide any further data of the remaining USUV RNA-positive patients as they were admitted to other district hospitals of the province of Modena. Only CSFs or serum samples of these patients were sent to the AOUP of Modena that, indeed, may serve as diagnostic center or the other hospitals of the area. In all patients examined (n= 915), USUV RNA prevalence (1.1%; CI 95%: 0.06%-2.0%) was significantly higher ($p < 0.05$) than the prevalence of WNV (0%; CI 95%:0%-0.33%).

Bird and mosquito samples

USUV RNA was detected in 12 blackbirds (*Turdus merula*) collected in the Marche region and in 1 pheasant (*Phasianus colchicus*), 1 Eurasian jay (*Garrulus glandarius*) and 1 common starling (*Sturnus vulgaris*), collected in the Emilia-Romagna region. One pool of *Culex pipiens* was also found positive for USUV RNA. The pool originated from the municipality of Ancona (Marche region).

Analysis of the sequences

A dataset of 122 partial NS5 gene nucleotide sequences was analysed. The dataset included 25 sequences produced in this study: 10 from USUV strains detected in human samples and 15 originating from animals and vectors. Furthermore, 96 partial NS5 sequences of all USUV strains publicly available were retrieved for the analysis. Accession numbers are showed in S1 Table. Sequences of USUV strains detected in human samples had 97% nucleotide identity to the South African reference strain SAAR-1776 but higher identity (99-100%) to USUV strains isolated from birds and mosquitoes of different Italian regions. Partial NS5 sequences also shared a high nucleotide identity (99-100%) to Vienna 2001 and Budapest 2005 strains, representative of USUV strains identified in Central Europe. The phylogenetic analysis (Fig 1) performed on a portion of the NS5 encoding gene reveals that Italian strains of all origin and years cluster together with central-European strains.

Serological data

Of the 609 serum samples tested, 40 (P= 6.57%, CI 95%: 4.87% -8.82%) had USUV neutralising antibodies (Table 3A) whereas 18 (P=2.96%, CI 95%: 1.89% -4.62%) had WNV neutralising antibodies (Table 3B). Overall, in about 4 years of investigation in the 609 tested samples, the number of serum samples with USUV neutralizing antibodies was significantly higher ($p < 0.05$) to that of serum samples with WNV neutralizing antibodies.

DISCUSSION

The municipality of Modena is located in the Emilia-Romagna region, one of the areas of the Italian peninsula affected by intense flavivirus circulation. In the recent years human diseases caused by mosquito-borne viruses were increasingly reported in this region [22], and remarkably the first two human cases of USUV-associated encephalitis were diagnosed in 2009 in the local AOUP [13-14]. In this study we primarily tested CSF and serum samples for USUV-RNA collected during the period of the year with the highest presence of mosquitoes. If we take into account that CSF samples are normally collected from patients with suspected of viral encephalitis or meningoencephalitis, our data might be interpreted as a further confirm of the neuro-invasiveness of this virus. Moreover, this analysis revealed that the earliest USUV neuroinvasive infection detected in this study occurred in 2008, the same year of the first detection of WNV neuroinvasive disease (WNND) which instead occurred in a rural area between Ferrara and Bologna (Emilia-Romagna region), Italy [23]. Interestingly, in the period 2008-2009 a five-fold number of cases of USUV-neuroinvasive infections compared to the number of WNND cases were evidenced in this area. Therefore, if we sum the two cases of USUV infection described in 2009 in the province of Modena and the 8 cases discovered in this study, we obtain a total number of 10 cases while only two cases of WNND were notified between 2008 and 2009 [24].

USUV has been recently regarded to exist in multiple lineages with Senegal as possible origin for the progenitor of Central European epizootics [25]. Given the high percentage of nucleotide identity within all USUV strains detected in this study and with the homologous sequences of USUV strains Vienna 2001 and Budapest 2005, it can be assumed that the Italian strains, including the Italian USUV strains (MO_09_Hu

and BO_09_Hu) responsible for the two early human cases of encephalitis, are strictly related with strains that have been circulating in Central-Eastern Europe. They have been probably introduced in Italy by short distance migratory birds as previously suggested [6].

This study also highlights that USUV infection is not a sporadic event in humans, at least in the area of the municipality of Modena. The 6.57% of the tested patients (N=609) showed USUV antibodies, a percentage significantly higher ($p < 0.05$) compare to that of WNV (2.96%). On the other hand, the value of WNV sero-prevalence obtained out of 609 samples is within the range obtained in other studies demonstrating the 0.7-0.8% in healthy blood donors and the 3.1% in farm workers [26].

USUV sero-prevalence represents an unprecedented finding in this area. Previous sero-epidemiological surveys in healthy blood donors indicated WNV as the prevalent (0.78%) flavivirus causing infection in humans while USUV was regarded as an occasional finding (0.23%) [27]. In this regard, the incongruity between our and previous data is probably due to the selection criteria of the sampled population. In our study, USUV sero-prevalence was calculated in a more representative population with respect to that of healthy blood donors. Our serum samples were indeed collected from inpatients/outpatients with a broad range of medical histories including sick and healthy subjects. These subjects form a more representative subset of the general population, because of their heterogeneity, compared to a selected, more homogenous and steadily monitored population as that of healthy blood donors. In conclusion, this study highlights the need to face USUV and WNV with the same preventive approach. It is therefore rational to set up an efficient surveillance system, mainly in the areas with sustained virus circulation in animals, in order to implement measures to control the spread of the infection through diagnostic monitoring as already occurs in the Emilia Romagna Region since 2012 [27] including also blood [28] and organ donors. Eventually, this study perfectly demonstrates the effectiveness of the One Health approach for the analysis of human diseases with clear implications of animals and insects.

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CONFLICT OF INTEREST DISCLOSURE

The authors disclose any potential financial and personal relationships with other people or organizations that could inappropriately influence their work.

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Legend

Figure 1: Phylogenetic analysis. Maximum Likelihood tree inferred from multiple nucleotide sequence alignment of partial NS5 gene sequences of the USUV strains detected in this study. The option “Display only topology” has been used for graphical representation of the analysis. All homologous USUV sequences publicly available were recruited for the analysis. The tree was built with MEGA6 [20] using the K2+I (Best DNA model) as substitution model. Branch support was assessed with 500 bootstrap replicates. Recent German USUV sequences included in the analysis were provided with accession numbers. On the other hand, for more details regarding the sequences obtained in this study (from humans, birds and insects) and reference sequences, the reader is invited to see Table S1. Orange dots, German strains (2010-2014); red dots, African lineage 1; Blue squares and magenta diamonds, African lineages; purple dots, Central-European reference USUV sequence strains; green triangles, sequences obtained in this study from humans, birds and insects. All Italian USUV sequences, including those obtained in this study, belong to the major European cluster which may include additional lineages [25] according to the geographical areas of detection and to the emergence of local variants.

Table 1. Cerebrospinal fluid (CSF) and serum samples tested for Usutu virus (USUV) (A) by RT-PCR (RT_{USUV}) and for West Nile virus (WNV) (B) by quantitative real time RT-PCR ($qPCR_{WNV}$)

A

Samples	RT_{USUV}		Total samples tested	Positive (%)	Confidence interval 95%
	Positive	Negative			
CSF	8	298	306	2.61	1.35-5.07
Sera	2	607	609	0.33	0.10-1.18

B

Samples	$qPCR_{WNV}$		Total samples tested	Positive (%)	Confidence interval 95%
	Positive	Negative			
CSF	0	306	306	0	0-0.97
Sera	0	609	609	0	0-0.49

Table 2. Anamnestic and clinical data of 4/8 patients showing USUV RNA. M, male; CSF, cerebrospinal fluid

Year	Sample	Age	Sex	Matrix	Municipality and Region	Anamnestic and clinical data
2008	MO1_08_Hu	40	M	CSF	Modena (Emilia Romagna)	Meningoencephalitis, Chronic liver disease
2008	MO2_08_Hu	73	M	CSF	Modena (Emilia Romagna)	Meningoencephalitis, Chronic Obstructive Pulmonary Disease, Diabetes
2009	MO7_09_Hu	54	F	CSF	Modena (Emilia Romagna)	Acute encephalitis and polyneuritis, hypertension
2008	MO8_08_Hu	67	F	CSF	Modena (Emilia Romagna)	Acute encephalitis, Aortic and Mitral insufficiency

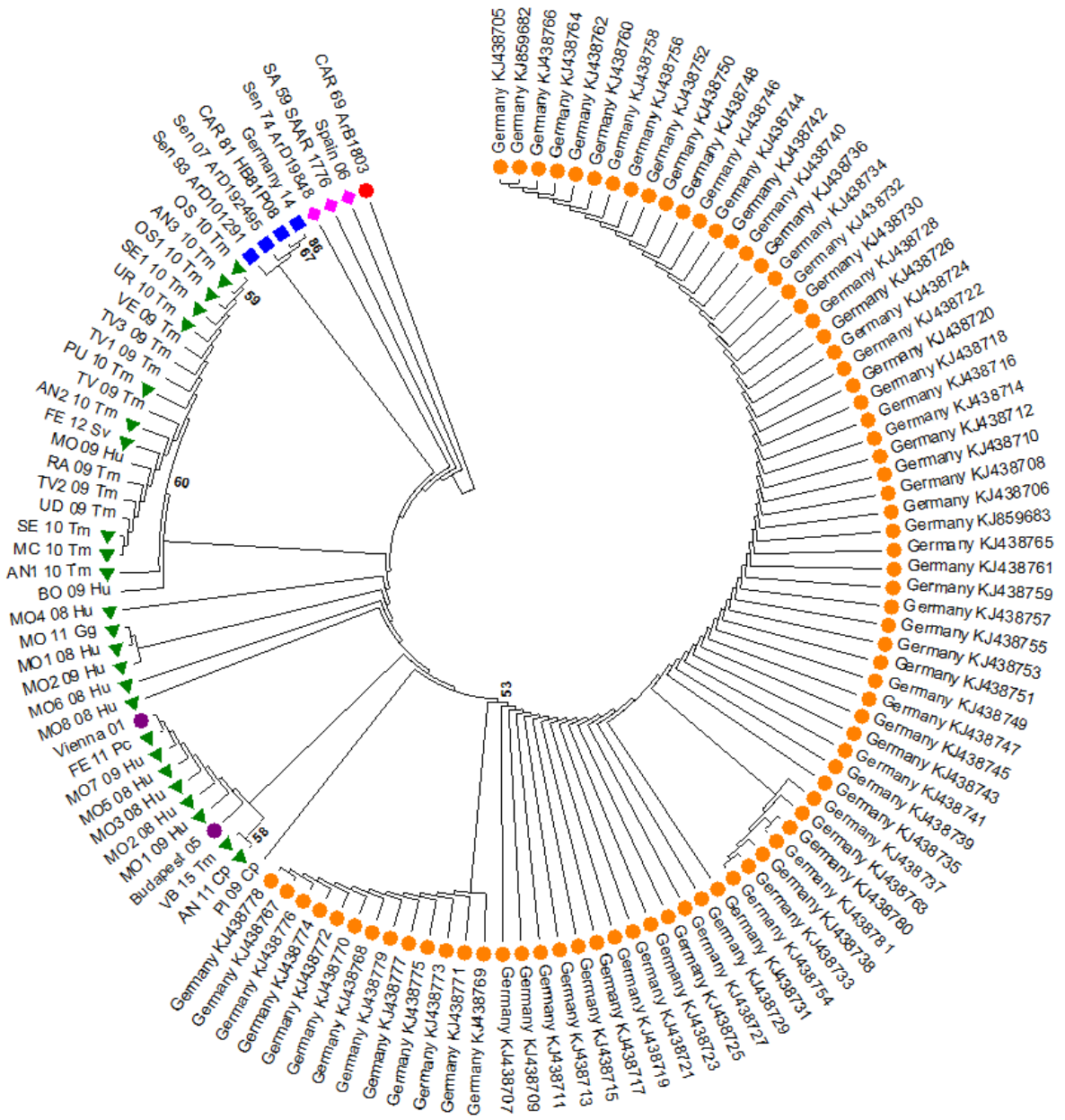
Table 3. Serum samples tested for the presence of Usutu virus (USUV) (A) and West Nile virus (WNV) (B) neutralizing antibodies.

A

Year	Serum Neutralisation		Total tested samples	Positive (%)	95% Confidence interval
	Pos.	Neg.			
2008	14	230	244	5.74	3.47-9.40
2009	21	285	306	6.86	4.55-10.27
2010	4	41	45	8.89	3.62-20.79
2011	1	13	14	7.14	1.66-31.95
Total tested	40	569	609	6.57	4.87-8.82

B

Year	Serum neutralisation		Total samples tested	Positive (%)	Confidence interval 95%
	Pos.	Neg.			
2008	5	239	244	2.05	0.90-4.70
2009	11	295	306	3.59	2.04-6.32
2010	1	44	45	2.22	0.53-11.53
2011	1	13	14	7.14	1.66-31.95
Total tested	18	591	609	2.96	1.89-4.62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