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Does *Aedes albopictus* (Diptera: Culicidae) play any role in Usutu virus transmission in Northern Italy? Experimental oral infection and field evidences

Arianna Puggioli[a,c], Paolo Bonilauri[b], Mattia Calzolari[b], Davide Lelli[b], Marco Carrieri[a], Sandra Urbanelli[d], Dubravka Pudar[e], Romeo Bellini[a,*]

[a] Centro Agricoltura Ambiente "G. Nicoli", Medical and Veterinary Entomology Department, Via Argini Nord, 3351, 40014 Crevalcore, BO, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "B. Ubertini" Brescia, Italy
[c] Dipartimento di Scienze Agrarie, Università di Bologna, Viale G. Fanin 42, 40127 Bologna, Italy
[d] Department of Environmental Biology, Sapienza University of Rome, Rome, Italy
[e] Laboratory for Medical and Veterinary Entomology, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000, Novi Sad, Serbia

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ABSTRACT

This study evaluated the vector competence of *Aedes albopictus* in transmitting USUV after oral infection under laboratory conditions. *Ae. albopictus* showed a low vector competence for USUV, although the positive body sample found with a very high number of viral copies at one week post infection indicates that a replication in the mosquito body can occur, and that USUV can escape the midgut barrier. Field data from an extensive entomological arboviruses surveillance program showed a relevant incidence of *Ae. albopictus* USUV positive pools in the period 2009–2012 while all pools were negative from 2013 on. No conceivable explanation regarding this field evidence was addressed, suggesting that attention must be paid to the trend of development of this vector-pathogen association, being aware of the potential rapid arbovirus' adaptation to new vectors, to prevent possible new disease's emergence.

1. Introduction

Usutu virus (USUV) is a mosquito-borne flavivirus, discovered in South Africa in 1959 (Ashraf et al., 2015), belonging to the Japanese encephalitis virus serocomplex and thus closely related to Japanese Encephalitis Virus (JEV), Murray Valley encephalitis virus (MVEV) and West Nile Virus (WNV) (Calisher and Gould, 2003). The first evidence of USUV circulation in Europe dates back to 2001 among blackbirds in Austria (Chvala et al., 2007), but evidences were found that the virus was already circulating in birds since 1996 (Weissenböck et al., 2002, 2013). Evidences of USUV circulation in Germany and Spain were found in 2012 (Allering et al., 2012; Höfle et al., 2013) and later in Croatia (Santini et al., 2015), The Netherlands (Rijks et al., 2016), France (Lecollinet et al., 2016). In 2016 a large epidemic was reported in birds across Belgium, The Netherlands, Germany and France (Cadar et al., 2017). In 2009 the first human cases of meningoencephalitis related to USUV infection occurred in Italy (Pecorari et al., 2009; Cavrini et al., 2009). In Italy, since its appearance, USUV continued to circulate among human population, as demonstrated by Gaibani et al. (2012) who detected Usutu-virus-specific IgG in blood donors from northern Italy and Cavrini et al. (2011).

In the life cycle of USUV, many mosquito species may act as vectors and many bird species as amplifying hosts. Mosquitoes can incidentally transmit the virus to humans and horses, while its isolation from bats has been recently reported in Germany (Cadar et al., 2014).

Among the numerous mosquito species in which the virus was isolated/detected (*Aedes albopictus*, *Ae. caspius*, *Anopheles maculipennis*, *Culex pipiens*, *Cx. neavei*, *Cx. perexiguus*, *Cx. perfuscus*, *Coquillettidia aurites*, *Mansonia africana*) *Cx. pipiens* is considered the most common vector (Ashraf et al., 2015). *Cx. neavei* is the only mosquito species whose vector competence for USUV has been investigated specifically (Nikolay et al., 2012) and therefore, vector competence studies involving other mosquito species should be done to determine their ability to transmit the virus.

In Northern Italy, the main vector species is considered *Cx. pipiens*, but also *Ae. albopictus* could be involved in the transmission cycle (Calzolari et al., 2010). *Ae. albopictus* is an invasive species rapidly adapting to temperate regions, already known for being a vector of dengue and other arboviruses, like chikungunya and Zika (Urbanelli et al., 2000; De Lamballerie et al., 2008, Bellini et al., 2012; Porretta

* Corresponding author.
E-mail address: rbellini@caa.it (R. Bellini).

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et al., 2012; Wong et al., 2013). Its known high anthropophily could favor the implication in USUV transmission cycles involving humans, as many *Ae. albopictus* pools sampled through the surveillance program of the Emilia-Romagna region (Northern Italy) tested positive to the USUV (Calzolari et al., 2012, 2013).

Our work dealt with the assessment of the vector competence of *Ae. albopictus* in transmitting USUV after oral infection under laboratory conditions.

2. Materials and methods

2.1. Laboratory experimental infection with USUV

The study was conducted in 2012 in a BSL-3 laboratory, in a climate-controlled chamber (28 ± 1 °C; 80% RH; 14:10 L:D), using *Ae. albopictus* females originated from wild collected eggs obtained from the Emilia-Romagna regional monitoring network (RER F_1 strain). Larvae were reared under controlled conditions and fed with a diet consisting of bovine liver powder, tuna meal and vitamin mix (Puggioli et al., 2016). 15 h before the oral infection, the sugar feeder was removed from the cage to increase female blood feeding.

Three different virus isolates were tested: USUV1 obtained from an infected blackbird (*Turdus merula* L.) collected in September 2011 in Ferrara (IZSLER internal code 231247/2011); USUV2 and USUV3 obtained from infected *Cx. pipiens* females collected in August 2011 (IZSLER internal codes 220921/2011 and 208576/2011 respectively) in the Emilia-Romagna regional West Nile surveillance plan (Calzolari et al., 2012, 2013). Complete genomes of these strains were obtained (Calzolari et al., 2017) and deposed in GenBank database (accession numbers U1: KF055442, U2: KF055441, U3: KF055440). The three isolates were passaged six times in Vero cells before being used in the study. The initial titers of the strains were $2*10^{7.5}$, $2*10^{7.5}$ and $2*10^{7.9}$ $TCID_{50}$/mL for USUV1, USUV2 and USUV3 respectively. For each virus isolate an infected blood meal was prepared by mixing mechanically defibrinated swine blood with the culture virus suspension at a 1:2 ratio (virus: blood). Therefore, for each treatment, 10 mL of virus suspension and 20 mL of swine blood were employed obtaining the following final virus titers: $0.66*10^{7.5}$ $TCID_{50}$/mL for USUV1; $0.66*10^{7.5}$ $TCID_{50}$/mL for USUV2; $0.66*10^{7.9}$ $TCID_{50}$/mL for USUV3. A non-infected blood meal, used as control, was prepared by mixing mechanically defibrinated swine blood with the culture medium used for the virus suspension (MEM plus 10% FBS), maintaining the same proportion (1:2) used for the infected blood meal. For each virus preparation 100, six-seven days old, females were exposed to the infected blood. The femaleswere previously kept in a cage together with the males to allow mating. The blood meals were offered by a unit feeding device, thermostated to maintain the blood suspension at 37 ± 1 °C, for 1 h (Bellini et al., 2012). For each treatment and the control, two replicates were performed. Five females and five males from the initial population were tested before the start of the experiment to assess for the absence of the USUV and other possible Flaviviruses.

2.2. Mosquito processing

After the exposure to the blood meal, the non-engorged females were removed from the cages, while the engorged females were maintained in the climate chamber. The number of dead females at the end of the exposure period was registered.

A sample of both infected and non-infected blood was taken and stored in dry ice to be checked for the presence/absence of the virus.

Pools of females (5–10 individuals) were removed from each cage at different post infection periods (pi from now on) (T0 = 5 h pi; T1 = 48 h pi; T2 = 96 h pi; T3 = 1 week pi; T4 = 2 weeks pi) for virus analysis. Females were individually anesthetized by chilling with ice, and processed under the stereomicroscope, as follows:

- Legs were carefully removed and placed in a cryovial.
- The female proboscis was inserted into a 1–5 µL capacity microcapillary tube (BLAUBRAND® IntraMARK, Wertheim, Germany) containing 1 µL of immersion oil (Merck, Darmstadt, Germany). The female was allowed to salivate for 45 min and the saliva was collected. Each microcapillary tube was then observed for saliva presence at the stereomicroscope and presence/absence of drops of saliva were recorded. The terminal part of the microcapillary containing the oil and the saliva was transferred to a cryovial containing 300 µl Eagle minimal essential medium (MEM).
- After saliva collection, each female body without legs was placed into a cryovial.
- The cryovials were stored in liquid nitrogen.

2.3. Virus detection

RNA extraction was performed from blood, mosquito saliva, legs and bodies by using TRIzol®LS Reagent (Invitrogen, Carlsbad, CA), and the total RNA extracted was suspended in 30 µl of DNAse/RNase free PCR grade water; the extracted RNA was retro-transcribed in cDNA according to a two-step PCR protocol, briefly cDNA synthesis was achieved using random hexamer (Roche Diagnostics, Mannheim, DE) and SuperScript® II Reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. The presence and the number of USUV copies were assessed by amplifying the cDNA obtained according to the protocol described in Cavrini et al. (2011). Also isolation of the virus was attempted from half of the mosquito saliva samples through inoculation on Vero cells. Briefly, samples were inoculated in confluent monolayer of Vero cells (African green monkey kidney cells), incubated at 37 °C with 5% CO_2 and observed daily for 7 days to highlight the development of cytopathic effect, in absence of this effect, the cryolysates were sub-cultured twice into fresh monolayers. The virus titers measured in the swine blood and in the swine blood added with MEM + FBS10% gave negative result both before and after the blood feeding. The virus titers measured just before blood meal resulted 6.8, 6.5 and 6.8 TCID50/mL for U1, U2 and U3 respectively. The virus titers measured in the blood meal after female feeding resulted 6.2, 5.5 and 6.5 TCID50/mL for U1, U2 and U3 respectively in replica A; while resulted 6.2, 6.5 and 6.2 TCID50/mL for U1, U2 and U3 respectively in replica B.

2.4. 2009-2015 USUV surveillance in Emilia-Romagna, Northern Italy

An integrated arbovirus surveillance plan has been implemented from 2009 in the Emilia-Romagna region covering an area of approximately 12,000 km^2. The north-west and central areas of the region are highly industrialized and intensively cultivated, while the eastern part comprehends large natural wetlands and the Po River Delta, facing the Adriatic Sea (Valli di Comacchio). More details can be found in Calzolari et al. (2012) and Bellini et al. (2014).

In the period 2009–2011, mosquitoes were collected only by means of modified CO_2–baited traps (a prototype developed by Centro Agricoltura Ambiente) (Bellini et al., 2002), while in the following years gravid traps (John W. Hock Company, Gainesville, Florida) were also employed. Details on the type and number of the traps and their allocation in urban areas in the seven-year study are reported in Table 1. Traps were positioned to cover the whole area under

Table 1
Mosquito surveillance traps in the seven-year study.

Year	2009	2010	2011	2012	2013	2014	2015	2016
No. CO_2 traps	74	110	90	88	76	72	72	77
No. Gravid Traps	0	0	0	8	16	16	16	16
% traps in urban areas	39.2	36.4	44.4	37.5	43.4	45.8	40.7	39.8

Table 2
Number of positive bodies out of the number of tested bodies for the three virus under investigation (in brackets the mean ± s.d. Ct value observed).

	USUV 1 (N = 73)		USUV 2 (N = 71)		USUV 3 (N = 70)		Negative control (N = 71)	
	a	b	a	b	a	b	a	b
5 h	4/5 (29.6 ± 2.5)	5/5 (28.0 ± 1.9)	5/5 (28.7 ± 2.8)	4/5 (27.3 ± 1.1)	3/5 (27.5 ± 1.2)	4/5 (32.3 ± 1.4)	0/5	0/4
48 h	2/5 (32.3 ± 0.3)	0/5	0/5	0/5	0/5	1/5 (34.7)	0/5	0/5
96 h	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10
1 week	0/8	0/10	1/6 (20.4)	0/10	0/6	0/10	0/7	0/10
2 weeks	0/10	0/10	0/10	0/10	0/10	0/9	0/10	0/10

surveillance. All traps were georeferenced and worked biweekly from approximately 5:00 P.M. to 9:00 A.M. Mosquitoes were identified to species level and pooled according to date, location and species, with a maximum of 200 individuals per pool (Sutherland and Nasci, 2007); afterwards, all pools were frozen at −80 °C. Detection method for USUV presence in mosquito pools are described in Calzolari et al. (2012). Based on the results of the PCR analyses of *Ae. albopictus* and *Cx. pipiens* pools, in the period 2009–2016 the maximum likelihood estimation (MLE) indice were calculated for both species by the software PooledInfRate, version 4.0 (Biggerstaff 2009).

3. Results

3.1. Laboratory experimental infection with USUV

The PCR analyses on a sample of males and females before the experiment showed the absence of USUV, and the same result was found for the controls (females fed on non-infected blood). At five hours pi, all of the three virus strains were present in the female bodies, and 25 out of 30 females tested virus-positive (Table 2). At 48 h pi, the number of virus-positive bodies decreased to8 out of 30, with any body females fed on the USUV2 infected blood meal was found positive. At the following check, at 96 h pi, none of the samples tested positive; at one week pi only one female was found positive at the PCR analysis to the USUV2 virus, while at two weeks pi no PCR positive body was found.

The PCR positive sample found one week pi showed a high number of viral copies, as it was found positive at Ct20, but, in contrast, the respective legs and saliva samples were found negative at the PCR analysis.

All the legs and saliva samples, included those obtained from females whose body tested positive for the virus presence, were found negative.

The attempt to isolate the virus from the body and from the saliva of the female which tested PCR positive at one week pi was not successful.

3.2. 2009-2016 USUV surveillance in Emilia-Romagna, Northern Italy

Table 3 summarizes the main results of the monitoring program in the period 2009–2016. As expected, the most captured species was *Cx. pipiens* (ranging from 81.4% in 2009 to 96.0% in 2014), being the regional plan primarily oriented to WNV surveillance. *Ae. albopictus* abundance resulted in the range 0.12–1.4%. Table 4 reports the MLE values calculated as a regional seasonal average for *Ae. albopictus* and *Cx. pipiens*. Surprisingly, while MLE values resulted quite stable in *Cx. pipiens* indicating a similar virus circulation in the whole period (mean seasonal MLE values in the range 0.23–0.54), in *Ae. albopictus* MLE resulted higher than in *Cx. pipiens* in the period 2009–2012 (MLE values in the range 1.24–3.50) while no more USUV positivity were found in *Ae. albopictus* in the period 2013–2016.

4. Discussion

The vector competence study conducted by oral administration to *Ae. albopictus* females of blood meals with three USUV isolates at doses in the range $0.66*10^{7.5}$–$0.66*10^{7.9}$ TCID50/mL showed a very low competence. These observations require further investigations possibly using other vector populations, virus strains and dosages.

Interestingly Nikolay et al. (2012) using initial virus titer $1.8*10^{8}$ PFU/mL observed in *Cx. neavei* a transmission rates of 81.3%, while at the lower titer of $2.0*10^{7}$ PFU/mL the transmission rate drop to zero. The authors concluded that the infection rate is strongly dependent to the virus titer of the blood meal (Nikolay et al., 2012).

Fros et al. (2015) evaluated the vector competence of *Cx. pipiens* for USUV using a blood meal containing $2*10^{7}$ TCID50 observing 69% of females with infectious saliva and thus concluding that *Cx. pipiens* is highly competent for USUV.

In our opinion, being the viral titers we administered to *Ae. albopictus* similar when compared to the initial titers used by Fros et al. (2015), but higher than those referred by Nikolay et al. (2012), we believe that the difference in the transmission rate measured at increasing time post infection cannot be due to inadequate viral titers in the blood meals.

Table 3
Total number of mosquito collected, most frequently captured species and frequency.

Year	2009	2010	2011	2012	2013	2014	2015	2016
Total mosquitoes collected	190,516	602,721	294,401	309,190	418,313	313,045	237,585	247,135
Most abundant species (%)	*Cx. pipiens* (81.4)	*Cx. pipiens* (92.4)	*Cx. pipiens* (88.4)	*Cx. pipiens* (82.2)	*Cx. pipiens* (92.3)	*Cx. pipiens* (96.0)	*Cx. pipiens* (88.7)	*Cx. pipiens* (91.1)
	Ae. caspius (15.4)	*Ae. caspius* (2.8)	*Ae. caspius* (8.2)	*Ae. caspius* (14.7)	*Ae. caspius* (4.5)	*Ae. caspius* (2.4)	*Ae. caspius* (8.7)	*Ae. caspius* (6.7)
	Ae. vexans (2.4)	*Ae. vexans* (4.1)	*Ae. vexans* (2.2)	*Ae. vexans* (1.8)	*Ae. vexans* (2.4)	*Ae. vexans* (0.6)	*Ae. vexans* (0.3)	*Ae. vexans* (1.0)
	Ae. albopic. (0.6)	*Ae. albopic.* (0.12)	*Ae. albopic.* (0.6)	*Ae. albopic.* (0.5)	*Ae. albopic.* (0.2)	*Ae. albopic.* (0.4)	*Ae. albopic.* (1.4)	*Ae. albopic.* (1.0)

Table 4
Maximum Likelihood Estimates (95% CI) of USUV in *Aedes albopictus* and *Culex pipiens* pools at the regional level.

Species	Year	Total N. mosquito analysed	N. Pools (1)	N. PCR + pools	MLE seasonal mean (Conf. Interval)
Ae. albopictus	2009	1227	108	2	1.64 (0.29–5.35)
	2010	738	45	2	2.74 (0.49–8.97)
	2011	1759	93	6	3.50 (1.44–7.26)
	2012	1613	123	2	1.24 (0.22–4.07)
	2013	958	78	0	0.00
	2014	1158	100	0	0.00
	2015	3317	190	0	0.00
	2016	1613	116	0	0.00
Cx. pipiens	2009	155,053	1259	54	0.36 (0.27–0.46)
	2010	397,785	2358	89	0.23 (0.18–0.28)
	2011	206,209	1360	46	0.23 (0.17–0.30)
	2012	193,288	1285	77	0.41 (0.33–0.51)
	2013	271,932	1865	88	0.33 (0.27–0.41)
	2014	257,660	2961	92	0.36 (0.29–0.44)
	2015	187,606	1838	99	0.54 (0.44–0.66)
	2016	204,805	2038	84	0.40 (0.32–0.50)

The RT-PCR on the body sample of only one female found positive at one week pi revealed a very high number of viral copies (Ct20). This finding indicates that a replication process in the female body had occurred.

The three strains were closely isolated in the same year and showed a close affinity, U1 and U2 had the same aminoacidic sequence and differed for 4 aminoacids to U3 (Calzolari et al., 2017). These differences did not seem to clearly affect the ability to infect *Ae. albopictus* since no difference was found in their infectivity, as the three viruses underwent the same rapid decrease in infectivity as time post infection increased.

Gaibani et al. (2013) described the complete genome sequence of the first USUV strain isolated from an immunocompromised patient with neuroinvasive disease, and found two aminoacid substitutions; one of them (D3425E) is highly remarkable since E3425 is highly conserved among the other USUV isolates that were not associated with human infection, while similar substitutions were observed in Japanese encephalitis and in West Nile viruses isolated from humans.

According to Nikolay et al. (2013), three specific amino acid substitutions were observed in the isolates from Europe which might alter virus secondary structures and influence its infectivity in vertebrate or mosquito cells, affecting the vertebrate host-vector relationship. These mutations might constitute adaptations to vector species abundant in Europe or influence the infectivity of host species. The authors suggest that these potential effects should be investigated in different cell culture systems and vector competence studies.

The rapidity of sequence change in RNA viruses means that RNA virus undergo rapid adaptation to changing host environments (Duarte et al., 1994) such as a shift in the vector species responsible for virus transmission to vertebrate hosts. The case of the E1-A226Vsubstitution in CHIKV that occurred in the spectrum of mutants was preferentially selected in *Ae. albopictus* in the mosquito midgut (Arias-Goeta et al., 2013). Interestingly, this particular adaptive substitution was speculated to have emerged independently at least three times during the last outbreaks (De Lamballerie et al., 2008; Tsetsarkin and Weaver, 2011). These findings bring new insight into the role of *Ae. albopictus* in contributing to the expansion of emerging arboviruses.

The arboviruses surveillance plan supported by the Emilia-Romagna Public Health Department showed that since the program was started in 2009 several *Ae. albopictus* pools were found positive to the USUV (Calzolari et al., 2010; Tamba et al., 2011). In the period 2009–2012(Table 5), the USUV MLE in *Ae. albopictus* was comparable or even higher than the MLE calculated for *Cx. pipiens*. The question why in 2013–2016 any USUV positive *Ae. albopictus* pools were found, remains unanswered. We have no possible explanation as any changes in the surveillance system was adopted between the two periods.

5. Conclusions

Our experiment showed that *Ae. albopictus* has a low vector competence for USUV. Nevertheless, in the field, the association between *Ae. albopictus* and USUV resulted common in the period 2009–2012, while disappeared in the period 2013–2016, without possible explanation. Being the emergence of arboviruses related to their ability to exploit new environments, for example a new host, thanks to the high mutation rate occurring during viral genome replication, the high incidence of *Ae. albopictus* USUV positive pools found through the arboviruses' entomological surveillance program requires that attention is paid to the trend of development of this new relationship.

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