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SHORT COMMUNICATION

Evaluation of vector competence for West Nile virus in Italian *Stegomyia albopicta* (=*Aedes albopictus*) mosquitoes

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Abstract. West Nile virus (WNV) is a zoonotic arboviral pathogen transmitted by mosquitoes in a cycle that involves wild birds as reservoir hosts. The virus is responsible for outbreaks of viral encephalitis in humans and horses. In Europe, *Culex pipiens* (Diptera: Culicidae) is considered to be the main vector of WNV, but other species such as *Stegomyia albopicta* (=*Aedes albopictus*) (Diptera: Culicidae) may also act as competent vectors of this virus. Since 2008 human cases of WNV disease have been reported in northeast Italy. In 2011, new areas of southern Italy became involved and a first outbreak of WNV lineage 1 occurred on the island of Sardinia. On the assumption that a potential involvement of *St. albopicta* in WNV transmission cannot be excluded, and in order to evaluate the competence of this species for the virus, an experimental infection of an *St. albopicta* laboratory colony, established from mosquitoes collected in Sardinia, was carried out. The results were compared with those obtained in a colony of the main vector *Cx. pipiens*. The study showed *St. albopicta* collected on Sardinia to be susceptible to WNV infection, which suggests this Italian mosquito species is able to act as a possible secondary vector, particularly in urban areas where the species reaches high levels of seasonal abundance.

Key words. *Aedes albopictus*, *Stegomyia albopicta*, vector competence, West Nile virus, Sardinia.

West Nile virus (WNV) is a *Flaviviridae*, genus Flavivirus, and was isolated for the first time in Uganda in 1937. The virus is an emerging mosquito-borne RNA virus that can infect the central nervous system in various host species and cause severe neurological disease. The enzootic transmission cycle involves mosquitoes and birds (Gray & Webb, 2014); horses and humans are considered incidental hosts, in which the virus can cause meningoencephalitis. The virus has been responsible for sporadic cases of WNV neuro-invasive disease in humans and horses in northeastern Italy every year since 2008 (Barzon *et al.*, 2013). In 2011, cases were confirmed in regions that had been involved in previous years, and also in new areas of southern Italy (Calabria and Basilicata regions), and for the first time on the island of Sardinia, where an outbreak of WNV lineage 1 occurred in late summer, causing acute neurological disease in six hospitalized patients, four of whom died (Di Sabatino *et al.*, 2014).

In Europe, *Culex* spp. mosquitoes, and in particular *Culex pipiens*, are reported to be the main vectors of WNV and to play a primary role in both viral enzootic maintenance and epizootic transmission (Di Sabatino *et al.*, 2014). Other mosquito species, such as *Stegomyia albopicta* (=*Aedes albopictus*), are also

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considered potential competent vectors, although their involvement in viral transmission remains unclear (Tiawsirisup *et al.*, 2004, 2005). Since the first record of *St. albopicta* in 1990, the invasive 'Asian tiger mosquito' has become established throughout Italy (Romi *et al.*, 2009; ECDC, 2009). This peridomestic and anthropophilic species is considered an experimentally competent vector of at least 26 arboviruses, including WNV (Paupy *et al.*, 2009). Although this species has been found to be naturally infected with WNV in North America (Holick *et al.*, 2002; Turell *et al.*, 2005), very little is known about its real susceptibility and its ability to sustain WNV outbreaks. As *St. albopicta* is considered to be an opportunistic feeder (Paupy *et al.*, 2009), its role as a potential bridge vector for WNV must be taken into account.

The aim of this study was to experimentally investigate the susceptibility of a *St. albopicta* colony, originating from mosquitoes collected in Sardinia, to WNV lineage 1 isolated during the Sardinia outbreak. In addition, a comparison with the experimental infection results of an Italian *Cx. pipiens* laboratory colony was carried out.

The *St. albopicta* colony collected in Cagliari (Sardinia) and a parallel *Cx. pipiens* colony from Frascati (Rome Province, Latium region) were established in the insectarium of the Istituto Superiore di Sanità in Rome and maintained for several generations in a climatic chamber under the following environmental parameters: temperature 27 ± 1 °C; relative humidity (RH) 70%, and an LD 14 : 10 h photoperiod.

Firstly, in order to check that the two colonies were virus-free, 10 pools of about 20 specimens per pool for each species were analysed for WNV, and Chikungunya and Dengue viruses by real-time polymerase chain reaction (PCR) and by inoculation in Vero cells. All pools showed negative results.

Both *St. albopicta* and *Cx. pipiens* colonies were fed with an infectious blood meal via an artificial membrane feeding system. Oral infections were performed using 8–12 days old female mosquitoes and WNV strain Ma V3, belonging to lineage 1, isolated on Vero cells from the cerebrospinal fluid of a patient from the Sardinia outbreak in 2011 (Magurano *et al.*, 2012). Mosquito females were allowed to feed for 60 min through a pig intestine membrane covering the base of a glass feeder containing the virus–blood mixture (1 : 3), maintained at 37 °C by a warm water circulation system. After the infectious blood meal, engorged females were selected, transferred to cages and maintained on a 10% sucrose solution in a climatic chamber under the conditions reported above for 28 days.

To analyse the length of viral extrinsic incubation period, and the trend of viral growth in potentially infected females, groups of five to seven fed mosquitoes were collected at 0, 3, 9, 14, 21 and 28 days post-infection (p.i.). Each mosquito specimen was dissected by separating the legs and wings from the body. The entire proboscis was then inserted into a single quartz capillary filled with 3 µL of fetal bovine serum (FBS). One microlitre of 1% pilocarpine, an analogue of acetylcholine and a saliva-stimulant (Dubrulle *et al.*, 2009), prepared in phosphate-buffered saline (PBS) at 0.1% Tween 80, was applied on the thorax. After 30 min, medium containing the saliva was expelled under pressure from the capillary into a 1.5-mL tube containing 500 µL of mosquito diluent consisting of PBS, 20% heat-inactivated FBS, and a 1% penicillin/streptomycin/amphotericin B mix (Invitrogen Corp., Carlsbad, CA, U.S.A.; GIBCO Brl, Rockville, MD, U.S.A.). Viral titres were evaluated by testing for RNA presence by quantitative real-time PCR, performed with TaqMan primers and probe. Briefly, quantification of WNV in RNA samples was determined by comparing crossing point values with standard curves based on data acquired from 10-fold serial dilutions of virus stocks in which concentration was estimated by titration on Vero cells and expressed as $\log_{10}$ PFU/mL (Lanciotti *et al.*, 2000). For the experiment, an infected blood meal with a final concentration of 7.97 $\log_{10}$ PFU/mL was used (Tiawsirisup *et al.*, 2004, 2005; Amraoui *et al.*, 2012). Infection (IR), dissemination (DR) and transmission rates (TR) in the *St. albopicta* and *Cx. pipiens* colonies were assessed by detection of viral positivity in the bodies, legs and wings, and saliva, respectively, of the fed females. Differences in IR, DR and TR between the two colonies were tested for significance ($P < 0.05$) using Fisher's exact test.

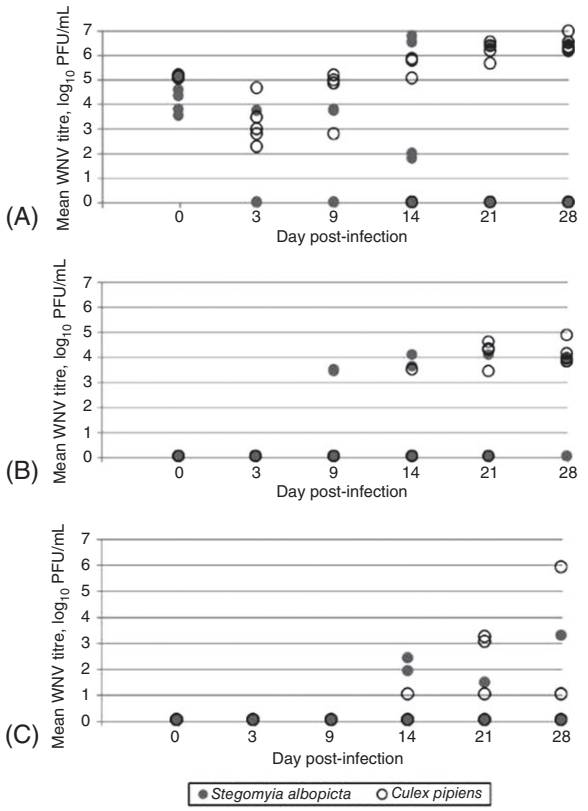

Fig. 1. Mean titres of infectious viral particles present in (A) bodies, (B) wings plus legs and (C) saliva of *Stegomyia albopicta* and *Culex pipiens* at different days post-infection with West Nile virus (WNV). *Cx. pipiens* and *St. albopicta* colonies were experimentally fed with infectious bloodmeals containing $10^{7.97}$ PFU/mL of WNV. At days 0, 3, 9, 14, 21 and 28 post-infection, groups of five to seven females were individually analysed.

As Fig. 1A and Table 1 show, all of the *St. albopicta* and *Cx. pipiens* bodies analysed at day 0 p.i. showed positive results with mean viral titres of 4.25 $\log_{10}$ PFU/mL and

Table 1. Mean West Nile virus (WNV) titres and infection (IR), dissemination (DR) and transmission rates (TR) in fed females of *Stegomyia albopicta* and *Culex pipiens* colonies.

	West Nile virus titre, $\log_{10}$ PFU/mL, mean ± SD					
	St. albopicta			*Cx. pipiens*		
Day p.i.	Body (IR*)	Legs + wings (DR†)	Saliva (TR‡)	Body (IR*)	Legs + wings (DR†)	Saliva (TR‡)
0	4.28 ± 0.63 (100%)	0 (0%)	0 (0%)	5.10 ± 0.06 (100%)	0 (0%)	0 (0%)
3	3.67 ± n/a (20%)	0 (0%)	0 (0%)	3.18 ± 0.90 (100%)	0 (0%)	0 (0%)
9	3.75 ± 0.04 (40%)	3.46 ± 0.04 (100%)	0 (0%)	4.49 ± 0.98 (100%)	0 (0%)	0 (0%)
14	4.24 ± 2.72 (80%)	3.82 ± 0.29 (50%)	2.13 ± 0.38 (50%)	5.52 ± 0.46 (60%)	3.98 ± n/a (33%)	1 ± n/a (33%)
21	6.33 ± n/a (20%)	4.07 ± n/a (100%)	1.43 ± n/a (100%)	6.16 ± 0.38 (80%)	4.15 ± 0.52 (100%)	2.39 ± 1.21 (75%)
28	6.40 ± n/a (20%)	3.85 ± n/a (100%)	3.28 ± n/a (100%)	6.41 ± 0.31 (71%)	4.12 ± 0.44 (100%)	1.7 ± 1.18 (60%)
Cumulative rate for days 9–28 p.i	(40%)§	(75%)	(50%)	(77%)§	(59%)	(41%)

*Number of positive bodies/number of tested fed females.
†Number of positive legs plus wings/number of positive bodies.
‡Number of positive saliva/number of positive bodies.
§Statistically significant difference ($P = 0.014$).
p.i., post-infection; SD, standard deviation; n/a, not applicable.

5.10 $\log_{10}$ PFU/mL, respectively, confirming the ingestion of infectious viral particles. These values decreased at day 3 p.i. as the direct effect of blood meal digestion, and then increased gradually from day 9 p.i. to day 28 p.i., reaching mean viral titres of 6.40 $\log_{10}$ PFU/mL and 6.41 $\log_{10}$ PFU/mL in *St. albopicta* and *Cx. pipiens*, respectively. Interestingly, differences in IR values between the two mosquito species were observed at each collection time. In particular, *St. albopicta* showed much lower IR values at day 21 p.i. (20%) and day 28 p.i (20%) in comparison with the *Cx. pipiens* colony at the same collection times (80% and 71%, respectively). Cumulative IR values, calculated as the total number of mosquitoes infected from day 9 to day 28 p.i., were 40% for *St. albopicta* and 77% for *Cx. pipiens*; this difference was statistically significant ($P = 0.014$) (Table 1). Disseminated infection started from day 9 p.i. in *St. albopicta*, and from day 14 p.i in *Cx. pipiens*, with mean viral titres fluctuating around 3–4 $\log_{10}$ PFU/mL until day 28 p.i (Fig. 1B). The presence of the virus in saliva was detected from day 14 p.i. in both colonies, with mean viral titres of 1–3 $\log_{10}$ PFU/mL (Fig. 1C). Cumulative DR and TR values from day 9 to day 28 p.i. were, respectively, 75% and 50% in *St. albopicta* ($P = 0.432$) and 59% and 41% in *Cx. pipiens* ($P = 0.678$) (Table 1). The high values of DR and TR in the *St. albopicta* colony were in agreement with findings in previous studies in which this mosquito species, feeding on blood meals with WNV titres of $\geq 10^{7.0}$ CID50s/mL, was more competent than *Cx. pipiens* (Tiawsirisup *et al.*, 2004, 2005; Vanlandingham *et al.*, 2007).

Although all mosquitoes in both colonies were found to be positive for WNV at time 0, immediately after the blood meal, a lower percentage of *St. albopicta* were found to be infected in the following days, which suggests the susceptibility of this species to WNV growth is lower than that of *Cx. pipiens*. Nonetheless, the present results clearly showed the tested *St. albopicta* colony to be capable of becoming infected and potentially transmitting WNV.

By contrast with findings in North America, where *St. albopicta* has been found to be naturally infected with WNV (Holick *et al.*, 2002; Turell *et al.*, 2005; Paupy *et al.*, 2009), and with the results of studies of its vector competence, which show high IR values after blood meals with titres of $\geq 10^{7.0}$ CID50s/mL (Tiawsirisup *et al.*, 2004, 2005), the low IR found in the Italian *St. albopicta* colony in the present study may be in agreement with the apparent absence of positive pools for WNV in natural *St. albopicta* populations (Calzolari *et al.*, 2010). However, the strong cumulative DR and TR values seem to confirm the susceptibility of *St. albopicta* to WNV infection and show that this species may potentially represent an additional vector in Italy. These factors, coupled with the mosquito's aggressive and opportunistic biting behaviour, may determine favourable conditions for WNV outbreaks, particularly in urban areas, in which the species reaches high levels of seasonal abundance as a result of the wide availability of breeding sites. However, further investigations with other Italian populations of *St. albopicta* are required to better define the role of this species in the cycle of transmission of WNV.

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