Vector Competence of *Aedes caspius* and *Ae. albopictus* Mosquitoes for Zika Virus, Spain

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We assessed the vector competence of *Aedes caspius* and *Aedes albopictus* mosquitoes in Spain for the transmission of Zika virus. Whereas *Ae. albopictus* mosquitoes were a competent vector, *Ae. caspius* mosquitoes were unable to transmit Zika virus. We also identified high levels of vertical transmission of Zika virus in *Ae. albopictus* mosquitoes.

Zika virus is an emerging arbovirus of the family *Flaviviridae* primarily transmitted by *Aedes aegypti* mosquitoes, but other *Aedes* species mosquitoes could be competent vectors (*1*). *Ae. aegypti* mosquitoes are absent from most countries in Europe (*2*), and the invasive *Ae. albopictus* mosquito and other native species could create novel epidemiologic scenarios for Zika virus. Indeed, *Ae. albopictus* mosquito populations from Europe are competent vectors for Zika virus (*3,4*). However, the vector competence for transmission of Zika virus of most mosquito species of Europe is currently unknown and may vary across virus strains and mosquito populations (*5*).

Although no autochthonous vectorborne Zika virus transmission has been reported in Spain, ≥316 imported cases of Zika virus have been confirmed (*6*). The confirmed cases, together with the presence of both the *Ae. albopictus* mosquito (*7*) and the native *Ae. caspius* mosquito (*8,9*) (a potential vector of chikungunya virus [*10*] and Rift Valley fever virus [*11*]), indicate a risk for Zika virus transmission in Spain. Accurately quantifying this risk requires evaluating the competence of these mosquito species for Zika virus.

We determined vector competence at different days postinfection (dpi) by exposing F0 generation of *Ae. caspius* mosquitoes (collected as larvae in Huelva, Spain, because we were unable to rear it under laboratory conditions) and F2 generation of *Ae. albopictus* mosquitoes (collected as eggs in Barcelona, Spain) to Zika virus through infectious blood meals. We used F8 generation of colonized populations of *Ae. aegypti* mosquitoes (collected in Poza Rica, Mexico) as a control population and Zika virus strains CAM (2010 Cambodia; GenBank accession no. JN860885) and PR (2015 Puerto Rico; GenBank accession no. KU501215), passaged 4 times on Vero cells and 2 times on C6/36 cells. We propagated on C6/36 cells for 4 days, and freshly harvested supernatant was mixed 1:1 with sheep blood (Colorado Serum Company, http://www.thepeakofquality.com) and 2.5% sucrose (*5*).

We offered to 4- to 7-day-old *Ae. albopictus* and *Ae. aegypti* female mosquitoes infectious blood meals containing either the CAM or PR strain at a final concentration of 7.6 $\log_{10}$ PFU/mL. Infection rates were determined by screening mosquitoes' bodies, dissemination rates by screening legs, and transmission rates by screening saliva, at 3 different time points (7, 14 and 21 dpi) using Zika-specific quantitative reverse transcription PCR including negative controls in each reaction (*12*) (Table 1; Appendix, https://wwwnc.cdc.gov/EID/article/25/2/17-1123-App1.pdf). We calculated Zika titers from standard curves on the basis of infectious particle standards created from matched virus stocks (*5*).

We further exposed 4- to 10-day-old *Ae. caspius* female mosquitoes to the PR strain as described. We conducted 3 independent trials using different Zika virus concentrations at different time points (7, 14, or 21 dpi) for each trial (Table 1; Appendix).

To determine the ability of *Ae. albopictus* mosquitoes to vertically transmit Zika virus, 4- to 7-day-old females were infected with Zika PR as described, and noninfectious blood meals were offered weekly after the first oviposition. We collected eggs laid in the second oviposition and hatched them for subsequent testing. We grouped second instar larvae in pools of 5 individuals and tested them for Zika virus (*13*). We estimated vertical transmission rate, measured as filial infection rate using the maximum-likelihood method (PoolInfRate version 4.0, https://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html) (*13*).

We performed generalized linear models with binomial error distribution and logit link function to assess the effect of mosquito species, virus strains, and dpi on the

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DOI: https://doi.org/10.3201/eid2502.171123

Table 1. Infection, dissemination, and transmission rates of mosquitoes experimentally infected with 2 Zika virus strains, Spain

Days postinfection	Mosquito species	Zika virus strain*	Blood meal titers, log_{10} PFU/mL	% Infected (total no.)	% Infected disseminating	% Infected transmitting
7	*Aedes aegypti*	CAM	7.6	24.2 (33)	75	12.5
		PR	7.6	61.8 (34)	38.1	0
	Ae. albopictus	CAM	7.6	90.5 (21)	42	10.5
		PR	7.6	97.0 (33)	31.3	0
	Ae. caspius	PR	7.7	21.4 (14)	0	0
14	*Ae. aegypti*	CAM	7.6	22.6 (31)	71.4	14.3
		PR	7.6	45 (40)	77.8	16.7
	Ae. albopictus	CAM	7.6	81.5 (27)	81.8	9.1
		PR	7.6	93.3 (30)	67.9	0
	Ae. caspius	PR	8.7	40 (25)	0	0
21	*Ae. aegypti*	CAM	7.6	35.7 (28)	100	40
		PR	7.6	56.3 (32)	88.9	38.9
	Ae. albopictus	CAM	7.6	94.4 (18)	82.4	23.6
		PR	7.6	96.2 (26)	96	36
	Ae. caspius	PR	7.6	18.5 (27)	0	0

*CAM, Zika virus 2010 Cambodia strain; PR, Zika virus 2015 Puerto Rico strain.

infection, dissemination, and transmission rates. We also considered the interactions between virus strain and dpi and between virus strain and mosquito species. We determined differences in mean viral titers between mosquito species, virus strains, and dpi in mosquito body, legs, and saliva using Kruskal-Wallis tests. Analyses were run in JMP version 9 (SAS Institute, http://www.jmp.com).

Infection rate was higher in *Ae. albopictus* than in *Ae. aegypti* mosquitoes, and Zika PR had a higher infection rate than Zika CAM. Dissemination rate increased with time (dpi) but was similar between mosquito species and Zika strains. Transmission rate also increased with time, and mosquitoes infected with Zika CAM showed a higher transmission rate than those infected with Zika PR. Transmission rate did not differ between *Ae. albopictus* and *Ae. aegypti* mosquitoes (Table 1, 2). Mean viral titers in bodies differed between mosquito species and Zika strains, with higher titers in *Ae. albopictus* compared with *Ae. aegypti* mosquitoes ($\chi^2 = 5.09$, df = 1; $p<0.02$) and higher titers for Zika PR compared with Zika CAM ($\chi^2 = 6.92$, df = 1; $p<0.009$). Mean viral titers in legs were similar for both Zika strains ($\chi^2 = 0.95$, df = 1; $p = 0.33$), but were higher in *Ae. aegypti* relative to *Ae. albopictus* mosquitoes ($\chi^2 = 9.53$, df = 1; $p<0.002$). Mean viral titers did not differ in saliva secretions between mosquito species ($\chi^2 = 1.7$, df = 1; $p = 0.19$) or Zika strains ($\chi^2 = 1.02$, df = 1; $p = 0.31$). We detected Zika virus infection in *Ae. caspius* mosquitoes at 7, 14, and 21 dpi, but detected no virus dissemination or transmission at any point (Table 1). Five larval pools of *Ae. albopictus* mosquitoes (29.4%; N = 17) were positive for Zika virus, with a filial infection rate of 72.2 (95% CI 27.6–156.1) and mean viral load of 2.5 log_{10} PFU/mL. This value equates to a ratio of 1:14.

Our results suggest *Ae. albopictus* mosquitoes in Spain are competent vectors of Zika virus at levels similar to *Ae. aegypti* mosquitoes. We detected Zika CAM in saliva earlier than Zika PR, which suggests that genetically variable strains may have different transmission potential (*5*). Although a similar transmission rate was found in *Ae. albopictus* mosquitoes from Spain and Italy (*3*), lower rates were measured in populations in France (*4*). In addition, *Ae. albopictus* mosquitoes from Spain could transmit Zika virus at 7 dpi, 4 days earlier than mosquitoes in Italy (*4*). These discrepancies may be explained by variation in vector competence between mosquito populations and virus strains (*5*). Although Zika virus can infect *Ae. caspius* mosquitoes, it is unable to escape the midgut and be effectively transmitted (*14*).

Zika virus is vertically transmitted by the population of *Ae. albopictus* mosquitoes in Spain at substantially higher rates than found in *Ae. albopictus* mosquitoes from New York and Italy (*4*,*13*) and for other flaviviruses (*15*). These results suggest that the ability of Zika virus to be transmitted vertically is highly population dependent and could contribute to maintenance of the virus in *Ae. albopictus* mosquitoes in Spain.

Our results confirm that populations of *Ae. albopictus* mosquitoes increase the risk for Zika virus transmission in

Table 2. Results of generalized linear models analyzing the variation in infection, dissemination, and transmission rates of Zika virus*

	Infection rate			Dissemination rate			Transmission rate		
Variable	χ^2	df	p value	χ^2	df	p value	χ^2	df	p value
Mosquito species	110.95	1	**<0.001**	2.08	1	0.15	2.37	1	0.12
Zika virus strain	10.43	1	**0.001**	1.28	1	0.26	4.91	1	**0.03**
dpi	0.15	1	0.70	39.61	1	**<0.001**	26.77	1	**<0.001**
Zika virus strain • dpi	1.17	1	0.28	1.34	1	0.25	6.70	1	**0.01**
Mosquito species • Zika virus strain	0.01	1	0.90	0.76	1	0.39	0.01	1	0.94

*Bold indicates significant effect; • indicates interaction between variables; dpi, days postinfection.

Spain. The high number of imported Zika virus cases and the rapid spread of *Ae. albopictus* mosquitoes contribute to the risk for autochthonous transmission of Zika virus. The risk for transmission by *Ae. caspius* mosquitoes, however, may be considered extremely low.

Acknowledgments

We thank A. Díez, M. Ferraguti, A. Pastoriza, and L. Gómez for helping during fieldwork. We thank members of the New York State Department of Health Arbovirus Laboratory staff for their assistance.

Projects CGL2012-30759 (BES-2013-065274) and CGL2015-65055P from MINECO supported this study.

About the Author

Dr. Gutiérrez-López is a researcher at Estación Biológica de Doñana interested in the study of vector competence of European mosquitoes for different vectorborne pathogens.

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Article DOI: https://doi.org/10.3201/eid2502.171123

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Appendix

Mosquito Collection and Rearing

More than 2,000 eggs of *Aedes albopictus* mosquitoes were originally collected in clapboards in Barcelona (northeastern Spain) in summer 2016; they were shipped to the New York State Department of Health (NYSDOH) Arbovirus Laboratory (Albany, NY, USA) and subsequently colonized. *Aedes aegypti* mosquitoes (provided by Gregory Ebel, Colorado State University) were originally collected in Poza Rica, Mexico, and subsequently colonized in the NYSDOH Arbovirus Laboratory.

Additionally, 2 shipments were sent to the NYSDOH Arbovirus Laboratory, each with ≈1,000 larvae of *Aedes caspius* mosquitoes collected in marshlands of the Huelva province (southern Spain) in summer and autumn 2016.

All mosquitoes were fed *ad libitum* with a 10% sugar solution and maintained under standard insectary conditions.

Oral Infection and Processing of Mosquitoes

All female mosquitoes were starved for 24 hours and subsequently fed with an infectious blood meal warmed to 37°C using a Hemotek feeding system (Discovery Workshops, http://hemotek.co.uk/).

F0 *Ae. caspius* female mosquitoes were infected with Zika virus 2015 Puerto Rico (PR) in 3 independent trials. Ninety-two *Ae. caspius* mosquitoes were exposed to ZIKV (blood meal titer = 8.7 $\log_{10}$ PFU/mL) in the first infection trial. Of those, 31 were completely engorged and the competence for the transmission of Zika virus was determined at 14 days postinfection (dpi).

In the second trial, 61 *Ae. caspius* mosquitoes were exposed to Zika virus (blood meal titer = 7.7 Log_{10} PFU/mL) and 14 of them were completely engorged. These mosquitoes were used to determine competence of Zika virus at 7 dpi. In the third trial, 65 *Ae. caspius* mosquitoes were exposed to ZIKV (blood meal titer = 7.6 Log_{10} PFU/mL) and 32 of them were completely engorged. These mosquitoes were used to determine competence of Zika virus at 21 dpi.

Approximately 2,500 F2 *Ae. albopictus* and 2,500 F8 *Ae. aegypti* mosquitoes were exposed to Zika virus PR (blood titer = 7.6 log_{10} PFU/mL) and Zika virus CAM (blood titer = 7.6 log_{10} PFU/mL). Approximately 150 mosquitoes of each species were engorged with infected blood. Mosquitoes were housed in independent containers and competence for Zika virus was determined at 7, 14, and 21 dpi.

RT-qPCR Protocol for Virus Detection

We used the protocol detailed by Lanciotti et al. (*1*). In brief, the protocol consisted in a RT-qPCR reaction containing 5 µL of extracted RNA as template, 13 µL 2x Master Mix Quanta QScript ToughMix Low ROX (Quanta BioScience; https://www.quantabio.com/), 0.2 µL 100 µM of the primer Zika 1086 (5′- CCGCTGCCCAACACAAG –3′), 0.2 µL 100 µM of the primer Zika 1162 (5′-CCACTAAYGTTCTTTTGCAGACAT-3′), 0.04 uL 25 uM Zika Probe, and 6.56 µL Millipore water. The Zika Probe used was 5Cy5/AGCCTACCT/TAO/TGACAAGCAGTCAGACACTCAA/3IAbRQSp/, where TAO is a proprietary internal quencher and the Iowa black (3IAbRQSp) is also a quencher. The reactions consisted in a first step at 50°C for 3 min, followed by a second step at 95°C for 10 min, and then 40 cycles of 5 s at 95 °C and 45 s at 60 °C.

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