Impact of Chikungunya Virus on *Aedes albopictus* Females and Possibility of Vertical Transmission Using the Actors of the 2007 Outbreak in Italy

Romeo Bellini¹*, Anna Medici¹, Mattia Calzolari², Paolo Bonilauri², Francesca Cavrini³, Vittorio Sambri³, Paola Angelini⁴, Michele Dottori²

1 Medical and Veterinary Entomology, Centro Agricoltura Ambiente “G. Nicoli”, Crevalcore, Bologna, Italy, 2 Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna, Brescia, Italy, 3 Unit of Clinical Microbiology, Department of Haematology and Oncology, Regional Reference Centre for Microbiological Emergencies (CRREM), S.Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy, 4 Emilia-Romagna Region, Public Health Service, Bologna, Italy

* E-mail: rbellini@caa.it

**Funding:** The work has been funded through the fund RFPS-2007-7-639191 of the Italian Ministry of Health managed by the Health Agency of the Emilia-Romagna Region. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**Competing Interests:** The authors have declared that no competing interests exist.

**Citation:** Bellini R, Medici A, Calzolari M, Bonilauri P, Cavrini F, et al. (2012) Impact of Chikungunya Virus on *Aedes albopictus* Females and Possibility of Vertical Transmission Using the Actors of the 2007 Outbreak in Italy. PLoS ONE 7(2): e28360. doi:10.1371/journal.pone.0028360

**Editor:** Christopher N. Mores, Louisiana State University, United States of America

**Received** July 12, 2011; **Accepted** November 7, 2011; **Published** February 27, 2012

**Abstract**

We investigated the impact of CHIKV strains on some *Aedes albopictus* (Skuse) reproductive parameters and the possibility of vertical transmission. Two strains were collected in the area where the epidemic occurred in 2007, one isolated from mosquitoes, the other one isolated from a viraemic patient. Different types of blood meals, either infected or non-infected, were offered to *Ae. albopictus* females, that were then analyzed at increasing time post infection. The virus titre, measured by two RT-PCR methods in the blood meals, influenced the rate of infection and the rate of dissemination of CHIKV in *Ae. albopictus* body. We found individual variability with respect to the infection/dissemination rates and their latency both considering the female’s body and appendages. The hatching rate was significantly lower for the eggs laid by the infected females than for the control eggs, while the mortality during the larval development (from first instar larva to adult emergence) was similar among the progeny of infected and non-infected female groups. Our findings seem to support the hypothesis that the vertical transmission is a rare event under our conditions, and that a certain time period is required in order to get the ovariolos infected. Field observations conducted during the Spring 2008 showed no evidence of the presence of infected overwintering progeny produced by *Ae. albopictus* females infected during the 2007 outbreak.

**Introduction**

Chikungunya virus (CHIKV) is an arbovirus of the family Togaviridae, which is a human pathogen and causes symptoms such as high fever, severe joint pain (arthralgia), marked weakness and may be accompanied by skin manifestations [1]. While the acute febrile phase is usually resolved in a few days, the joint pain may persist for weeks or months, causing serious economic and social impact [2].

The first known outbreak of CHIKV was described in 1952 in Tanzania [3,4] and several epidemics occurred in Asia and Africa. Between 2005 and 2007 a violent outbreak occurred in the Indian Ocean islands [5]. During the same years in many European countries, including Italy [6], CHIKV infection was diagnosed in travellers coming from epidemic areas. During the summer 2007, and for the first time in a temperate country, an epidemic of this tropical disease occurred in Italy [7] in Castiglione di Cervia and Castiglione di Ravenna (Emilia Romagna region, Northern Italy), which together form a single urban area.

The epidemic index case was identified as a person travelling from India (Kerala state) in June 2007. After the first outbreak, four secondary minor outbreaks originated in Cervia, Ravenna, Cesena and Rimini, totalling 248 cases confirmed by laboratory investigations, 142 of which were residents in the initial affected area.

The vector was identified as the mosquito *Aedes albopictus* (Skuse) [8]. The species has been well established in the area for at least 10 years, after its introduction to Italy in the 1990s through the importation of used tires [9].

* Aedes albopictus can easily adapt to urban environments, exploiting all kinds of artificial containers as larval habitats. It feeds on many vertebrates, including birds, reptiles and amphibians, but prefers mammals, and in particular humans [10]. Under laboratory conditions, its vector competence for many arboviruses, including 7 alphaviruses, 5 flaviviruses and 8 bunyaviruses has been demonstrated [11], together with its ability to transmit some of these viruses to the offspring (dengue, Japanese encephalitis, West Nile, and Yellow fever viruses) [12–13].

* Aedes albopictus is well established in several European countries [14] and its presence has been reported in several regions of the Mediterranean basin, like Israel [15], Lebanon and Syria [16]. Although *Ae. albopictus* is active all year-round in Southern Italy [17], in Northern Italy the species overwinters in the egg stage [18]. However, it has been recently reported the discovery of CHIKV infected larvae in nature in the island of La Reunion [19],

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supporting the hypothesis that infected mosquitoes may develop from eggs laid by infected females.

Data on vertical transmission under laboratory conditions are scarce and inconsistent. Mourya [20] found no evidence of CHIKV vertical transmission in *Ae. aegypti* L. and *Ae. albopictus*, while Hailin et al. [21] obtained some evidence of transovarial transmission in both species. Recently, Vazquez et al. [22], using CHIKV and *Ae. albopictus* strains from La Reunion, were unable to detect vertical transmission.

The purpose of this study was to investigate the possible vertical transmission of CHIKV in a strain of *Ae. albopictus* collected in Italy in the area where the epidemic occurred in 2007. The search for the virus presence was conducted in parallel by two Laboratories that applied different PCR methods. According to Turell et al. [23], when the female body tests positive for the search of the virus, the mosquito may be considered infected, while the detection of the virus in the legs indicate a disseminated infection. Therefore we tested mosquitoes for infection and dissemination of virus during three gonotrophic cycles. The CHIKV strain isolated in Italy was found to be homologous to the one isolated during the outbreak of CHIKV in India in 2007 [7], and is characterized by an alanine to valine mutation at position 226 in the E1 envelope glycoprotein [24,25], that increases the vector capacity of the Asian tiger mosquito with respect to this arbovirus [26].

**Materials and Methods**

**Mosquitoes**

*Aedes albopictus* females used in the experiment originated from eggs collected in the area of the 2007 CHIKV epidemic in Autumn and kept in laboratory at 28±2°C, 80% RH, 16:8 photoperiod. Larvae (F0) were fed on a diet consisting of dry cat food (Friskies®), yeast and dry fish food (Tetramin®). Once at the adult stage they were given a 10% sucrose solution and females were offered a defibrinated rabbit blood meal, provided through a thermostatically controlled device. A filter paper was placed into a beaker as an egg deposition substrate for females. A sample of the F1 adults was analyzed by RT-PCR to check for the absence of CHIKV. The experimental infection was then performed using adult females of the F1 generation.

**Virus**

Two CHIKV isolates obtained during the 2007 epidemic were tested in parallel: the strain A (ITA07-RA1) was isolated by the Zooprofylactic Institute of Lombardia and Emilia-Romagna (from now on IZSLER) from adult mosquitoes collected in Castiglione di Ravenna during the epidemic. The strain B (ITA7-BIO 07) was isolated at the Department of Microbiology, University Hospital S. Orsola-Malpighi of Bologna (from now on DMUNIBO), from a viremic patient. Both strains were passaged 5 times in Vero cells before being used in the study. The titre of the strains A and B were 10^{7.3} and 10^{9} TCID_{50}/mL, respectively. The sequence obtained from the mosquito isolate (GB EU244823) had a 99% identity with the 2006 and 2007 Indian isolates (GB FJ000076, FJ000066), and showed the E1-Ala226Val mutation.

The infectious meal consisted of 2/3 washed human erythrocytes and 1/3 viral suspension (volume) in DMEM cell culture medium (MMedical, Milan, Italy) mixed by a magnetic stirrer for 1 min just before the experiment.

For both the virus isolates, three suspensions of 6 mL each were prepared, containing three different virus titres:

- isolate A: 10^{5.3} TCID_{50}/mL (A1), 10^{6.3} TCID_{50}/mL (A2), 10^{7.3} TCID_{50}/mL (A3)
- isolate B: 10^{5.5} TCID_{50}/mL (B1), 10^{6.0} TCID_{50}/mL (B2), 10^{4.5} TCID_{50}/mL (B3)

**Mosquito oral infection**

The oral infection was performed at the BL3 laboratory of the Agriculture and Environment Centre “G. Nicoli” (from now on CAA). Twenty-four hours before the infection, groups of 60 females aged 4–5 days were isolated in Plexiglas cages (18×18×18 cm) without sugar supply. Three non-infected blood meals were prepared as control: mechanically defibrinated rabbit blood (R), human washed erythrocytes (HE), human washed erythrocytes mixed with the culture medium used for the virus suspension (HE-M). Two infected blood meals were prepared by adding to the HE-M the isolate A (HE-A) and the isolate B (HE-B). The control blood meals R, HE, and HE-M were offered to two groups of females, while the blood meals HE-A and HE-B were offered to three groups of females.

The blood meals were maintained at 37°C by means of a thermostatically controlled device, and were offered to the females for 30 minutes. After that period, all the non-engorged females were removed from the cages, and samples of 1–3 engorged females were collected from the cages with infected blood to determine the amount of virus ingested. The residual blood was recovered from the feeding devices to check for the virus presence by means of RT-PCR.

The remaining engorged females were left in cages provisioned with an oviposition substrate and had free access to a 10% sucrose solution, at 28.0±1.0°C, 80% RH and 16:8 photoperiod. They were blood fed again 6 and 14 days after the first blood meal, with non-infected rabbit blood meals in order to obtain a total of three oviposition cycles.

Before each blood meal, three females were collected from each cage, anaesthetized on ice, and their legs were separated from the body. Legs and body of each mosquito were then separately analyzed by RT-PCR, to check for the viral infection (presence of the virus in the abdomen) and virus dissemination (presence of the virus in the legs). At the end of the experiment (19 days after the first blood meal) all females were collected from the cages, and the body and legs of each mosquito were separately analyzed by RT-PCR.

The eggs obtained from the three gonotrophic cycles (F1G1, F1G2 and F1G3) were treated for hatching [27] and the larvae were reared by using the diet described above. The adults obtained were individually analyzed with RT-PCR.

**RNA extraction**

Viral RNA isolation was performed on an automated nucleic acid extractor, the NucliSENS easyMAG (Biomérieux, France). Females were individually homogenized in 2 mL of Lysis Buffer and mixed. After 10 min incubation at room temperature, the lysate was centrifuged at 3,000 rpm for 5 min to eliminate mosquito debris, and processed by the NucliSENS easyMAG instrument.

**PCR protocols**

The extracted RNA was processed following two different protocols: the two steps RT-PCR method [28] applied by the IZS-LER laboratory, and the one step RT-PCR method [29], used by the DMUNIBO laboratory.

**Two step Real Time RT-PCR.** The amplification target was 208 bp of the E1 gene, which codes for the structural envelope protein E1. cDNA synthesis was achieved by using SuperScript® II Reverse Transcriptase (Invitrogen, UK) according to the manufacturer instructions and random primer (examer). A total of 3 μL of cDNA...
was amplified by using LightCycler® FastStart DNA Master® plus HybProbe reaction mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer instructions with a final volume of 20 μL, containing 1 pmol/μL of each of the primers CHIK-F 5’-AACGTGTCTGCTTTACTAAAG-3’ and CHIK-R 5’-CGAATTTGCCAAGCTTCTCCT-3’, plus 0.2 pmol/μL of CHIK-P labelled probe FAM-5’-CCAAATTGTCCYGGTCTTCCT-3’, plus 0.2 pmol/μL of CHIK-P labelled probe TAMRA. One-step RT-PCR was carried out in a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) with the following cycling parameters: 55°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 30 s, and 72°C for 45 s then a cooling step of 40°C for 30 s. A standard calibration curve was constructed using a 10-fold dilution series of a plasmid containing the PCR amplicon as the insert (commercially acquired from TIBMOL® [Genova, Italy]). The assay showed linear results for 3 logs of CHIKV plasmid dilutions. The analytical sensitivity was of 100 copies per reaction. In each assay a sample of CHIKV RNA was amplified as positive control.

One-step RT-PCR. The amplification target was 127 bp of the E1 gene, which codes for the structural envelope protein E1. A 20 μL reaction volume contained 10 μL 2× reaction mix, 6.75 mM MgSO4, 0.8 μL RT/Taq (SuperScript® III Platinum, Invitrogen, UK) 0.44 μM of each primer (CHIK E1F 5’-TCGACCGGCCCTCTTCTTTAA-3’, CHIK E1R 5’-ATCGAATG-CAGCGCAGACT-3’), 1 μL labelled probe (CHIK E1P 5’-TAMRA and CHIK E1R primers into the pCR 8/GW/TOPO TA (Invitrogen, UK) 0.44 μL FastStart DNA Masterplus (Roche Diagnostics GmbH, Mannheim, Germany) 0.03) than group showed significantly higher fecundity in comparison to the other four groups (F (4,7) = 8.0 and 0.001). No statistically significant difference was found between the egg fertility of the HE-sA and HE-sB females (blood infected with the isolates A and B, respectively). The HE-sB females (but not the HEs-A females) showed lower egg fertility with respect to the R, HE and HE-M females (N = 54) (P<0.001). After the second blood meal, the percentage of blood fed females was higher for the HE group with respect to all the others (F(4,7) = 6.54 and P = 0.02), the fecundity did not show any difference among the five groups (F(4,7) = 1.93 and P = 0.21) while the fertility of the eggs was lower in the two groups that had the first blood meal on blood infected with both strains (F(4,7) = 12.8 and P < 0.001) (Table 1). After the third blood meal, no statistically significant difference in the percentage of blood fed females was found among the five treatments (F(4,7) = 0.97 and P = 0.51). The females of the HE group showed significantly higher fecundity in comparison to the other four groups (F(4,7) = 8.0 and P<0.001). No statistically significant difference was found between the egg fertility of the HE-sA and HE-sB females (blood infected with the isolates A and B, respectively). The HE-sB females (but not the HE-sA females) showed lower egg fertility with respect to the R, HE and HE-M females (F(4,7) = 9.99 and P<0.001) (Table 1). Detection of the virus in the progeny. Among the 101 females fed with infected blood we collected 14 females at DPI 0 for immediate analysis. From the 87 remaining females we obtained 689 adults (371 males and 318 females) and the adult production ranged from the 25.9% of the females blood fed on HE-sA1 to the 6.2% of the females blood fed on HE-sB3 (Table 3). On average, the number of adults per female was 2.1 ± 1.7 for the first gonotrophic cycle, 11.2 ± 4.9 for the second gonotrophic cycle, and 1.6 ± 2.5 for the third one. CHIKV was detected in one female and two males, all obtained during the second gonotrophic cycle. The female was derived from HE-sA1 cage, while the males were derived from HE-sB2 and HE-sB3 cages. In the control cages, from the 55 females fed with non-infected blood we obtained 5,930 adults (3,409 males and 2,521 females) and

Fecundity

The fecundity of the females blood fed with the whole rabbit blood was higher than that of the females fed on the other four kinds of erythrocyte-based blood meals (F(4,7) = 15.20 and P<0.001), among which no statistically significant difference was found (Table 1).
the percent production of adults ranged from 70.5% for the females fed with R1 to 41.2% for the females fed with HE-M2 (Table 4).

Comparison between the two PCR methods
Both the body and the legs of each mosquito were analyzed in parallel by the two RT-PCR methods, and the quantitative results, expressed in terms of number of viral copies obtained per microliter, showed a high positive correlation between the two methods of analysis (y = 27.958x + 0.894, R² = 0.76).

The one-step PCR was more sensitive than the two-step PCR, with differences in the range of 10–100 viral copies per microliter (1.16 ± 0.94 log10; mean ± SD) detected.

The quantitative analyses showed an increase in the number of viral copies detected in the body at 6 DPI in comparison with those obtained at 0 DPI, followed by a decrease at 14 and 19 DPI. The number of viral copies was lower in the legs with respect to the body, showing a tendency to decrease from 6 DPI to 14 and 19 DPI (Fig. 1).

Discussion
In our experiment, the virus titre in the blood meals influenced the rate of infection as well as the rate of dissemination of CHIKV in Ae. albopictus (Table 2). The values of dissemination of CHIKV observed in our study were much higher than previously reported for Ae. albopictus strains of different geographic origin by Turell et al. [30] (they used blood meals with 10^4.2–10^5.3 pfu/mL), but similar to the findings of Vazeille et al. [31] (they used blood meals

Table 1. Percentage of blood fed females, fecundity and fertility after the three blood meals (*).

| Blood meal | Type of the first blood meal | No. rep. | Mean | S.D. | N.K. | Mean | S.D. | N.K. | Mean | S.D. | N.K. |
|------------|------------------------------|----------|------|------|------|------|------|------|------|------|------|
| R          | whole rabbit erythrocytes based blood meal. HE, human erythrocytes based blood meal. HE-M, human erythrocytes plus virus culture medium. HE-sA, human erythrocytes plus viral strain A. HE-sB, human erythrocytes plus viral strain B. a, b, different letters indicate statistically significant differences at the probability level P = 0.05. NS, non significant. (*) Statistics are referred to comparisons among females blood fed with different kinds of blood in the same gonotrophic cycle. doi:10.1371/journal.pone.0028360.t001

Table 2. Results of the RT-PCR (two methods) performed on bodies and legs of virus-exposed females.

| Days Post Infection (DPI) | 0 | 6 | 14 | 19 |
|---------------------------|---|---|----|----|
| Females fed on infected blood | No. samples | No. PCR positive samples | No. PCR positive bodies | No. PCR positive legs | No. samples | No. PCR positive samples | No. PCR positive bodies | No. PCR positive legs | No. samples | No. PCR positive samples | No. PCR positive bodies | No. PCR positive legs |
| A1 | 3 | 3 | 3 | 3 | 1 | 3 | 2 | 1 | 10 | 10 |
| A2 | 3 | 3 | 3 | 3 | 2 | 3 | 1 | 0 | 4 | 0 |
| A3 | 3 | 3 | 3 | 1 | 1 | 3 | 0 | 0 | 13 | 3 |
| B1 | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 3 | 1 |
| B2 | 3 | 3 | 3 | 2 | 0 | 3 | 3 | 3 | 3 | 3 |
| B3 | 1 | 1 | 3 | 3 | 2 | 2 | 1 | 1 | 3 | 2 |

doi:10.1371/journal.pone.0028360.t002
with $10^7$ pfu/mL and tested also the CHIKV A226V mutated strain).

Strain B, despite the lower viral titres used in comparison to those used for strain A, was able to infect most of the females analyzed and to disseminate in the peripheral body parts, showing significantly higher infectivity capability. This finding is somewhat surprising, as both the viral isolates were obtained during the 2007 epidemic in Emilia-Romagna when a single index case was identified [7]. For this reason, it has to be taken into consideration that the observed differences in the infectivity capability could have been induced by the different laboratory procedures, confirming the importance of virus stock procedure’s quality during laboratory studies concerning vector biology [32].

The experimental design was not expected to provide data for the estimation of the extrinsic incubation period of CHIKV in *Ae. albopictus*, but we could observe a high rate of dissemination at 6 DPI, in particular at the highest titres tested, and a trend of increase of the dissemination rate at 14 and 19 DPI (Table 2).

We found individual variability with respect to the infection/dissemination rates and their timing in the female’s body and appendages, and our results were similar to studies conducted on other alphaviruses, such as Western equine encephalitis virus [33] and Eastern equine encephalitis virus [34].

At the highest virus titres tested (A1 and B1), qRT-PCR analysis found that the number of viral copies tend to increase in the female body at 6 DPI when compared with the values found at 0 DPI (Fig. 1). However, at 6 DPI, the virus has not disseminated well, as 7 out of 14 of the females with PCR-positive bodies had PCR-negative legs (Table 2). At 14 and 19 DPI, the number of viral copies in the bodies and legs did not increase, while an increased rate of dissemination was observed (Fig. 1). A similar trend in CHIKV titres in experimental infection studies in *Ae. albopictus* has been reported by Tsetsarkin et al. [26].

Some studies showed that pathogen infection may have detrimental impact on the vector reproductive capacity: *Anopheles gambiae* Giles and *An. stephensi* Liston, infected by the rodent malaria agent *Plasmodium yoelii nigeriensis*, showed reduced fecundity and fertility levels [35–36]. Other vector/pathogen systems showed a decrease in the number of larvae produced by the infected females, as demonstrated for *Culex tarsalis* Coquillett experimentally infected with West Nile virus [37] and Western equine encephalitis virus [38], as well as for *Culiseta melanura* (Coquillett) infected with the Alphavirus Eastern equine encephalitis virus [34].

In agreement with these findings, in our experiment the hatching rate was significantly lower for the eggs laid by the infected females than for the control eggs (Table 1), while the mortality during the larval development (from first instar larva to adult emergence) was similar among the progeny of infected and non-infected females groups.

Two possible explanations of the impact on the eggs fertility have been proposed: 1. a pathogenic action on the females, causing a decreased capacity to produce fertile eggs with a similar impact on the eggs fertility have been proposed: 1. a pathogenic action on the females, causing a decreased capacity to produce fertile eggs with a similar impact on

### Table 3. Mean percent of adults and number of adults per gonotrophic cycle from virus-exposed females.

| Type of blood | Mean % of adults (*) | Blood meal 1 | Blood meal 2 | Blood meal 3 |
|---------------|----------------------|-------------|-------------|-------------|
| HE-sA1        | 25.9                 | 75 (31/44)  | 108 (55/53) | 59 (27/32)  |
| HE-sA2        | 8.7                  | 26 (15/11)  | 43 (23/20)  | 4 (0/4)     |
| HE-sA3        | 17.2                 | 6 (1/5)     | 181 (80/101)| 0           |
| HE-sB1        | 19.6                 | 15 (7/8)    | 43 (19/24)  | 1 (1/0)     |
| HE-sB2        | 17.4                 | 55 (22/33)  | 51 (27/24)  | 0           |
| HE-sB3        | 6.2                  | 3 (2/1)     | 18 (8/10)   | 1 (0/1)     |
| Total         | 689 (371/318)        |             |             |             |

HE-sA, human erythrocytes plus viral strain A. HE-sB, human erythrocytes plus viral strain B. (*)Calculated on the total number of eggs laid by the females in the three gonotrophic cycles.

### Table 4. Number of adults obtained per gonotrophic cycle from non-infected control females.

| Type of blood | Mean % of adults (*) | Blood meal 1 | Blood meal 2 | Blood meal 3 |
|---------------|----------------------|-------------|-------------|-------------|
| R1            | 70.5                 | 987 (406/581)| 688 (309/379)| 355 (125/230)|
| R2            | 63.3                 | 1,140 (508/632)| 605 (245/360)| 403 (182/221)|
| HE1           | 45.1                 | 125 (48/77)  | 139 (45/94)  | 146 (85/61)  |
| HE2           | 64.4                 | 45 (271/8)   | 129 (56/73)  | 48 (18/30)   |
| HE-M1         | 48.2                 | 96 (33/63)   | 144 (47/97)  | 190 (88/102) |
| HE-M2         | 41.2                 | 142 (60/82)  | 294 (110/184)| 254 (99/155) |
| Total         | 5,930 (2,521/3,409)  |             |             |             |

R, whole rabbit erythrocytes-based blood meal. HE, human erythrocytes-based blood meal. HE-M, human erythrocytes plus virus culture medium. (*)Calculated on the total number of eggs laid by the females in the three gonotrophic cycles. (*) The total number of adults is followed by the ratio number of females/number of males.

doi:10.1371/journal.pone.0028360.t003
infected vs non-infected eggs [37]; 2. a pathogenic action on the infected egg/embryo only, thus strongly limiting the vertical transmission through selective mortality.

The depressive effect of the virus on the fertility of infected females (Table 1), described for other viruses during experimental infections [37], suggests that the CHIKV can increase the mortality of the infected egg/embryo. This mechanism may explain the low capacity of alphaviruses to go through transovarial transmission, already described in other studies [39–41]. We did not find any clear relationship between the occurrence of transovarial transmission and virus titres used to infect the females during the first blood meal. The virus titres tested in this study may be considered lower than the maximum viрemic titre observed in humans [42], thus requiring further investigations on freshly isolated viral strains at higher concentrations in the blood meal, to simulate the optimal transmission conditions for the virus.

Interestingly, in our study all of the three infected mosquitoes (2 males and 1 female) were obtained during the second gonotrophic meal, to simulate the optimal transmission conditions for the virus. The occurrence of such a low number of cases of vertical transmission (3 out of 689 adult mosquitoes, i.e. 0.43%) indicates that this is a rare event under our conditions, in agreement with the results of field observations conducted during the autumn 2007 and spring 2008, when about 8,000 larvae, collected in the Chikungunya affected area, were analyzed and tested negative, thus confirming that the vertical transmission in our scenario has to be considered a very rare phenomenon (Carrieri, unpublished data).

Acknowledgments

We thank Michael J. Turell for the key suggestions in planning the experiments and critical revision of the manuscript.

Author Contributions

Conceived and designed the experiments: RB PB PA VS MD. Performed the experiments: RB AM FC VS MD. Analyzed the data: RB PA MD. Contributed reagents/materials/analysis tools: RB FC MD. Wrote the paper: RB MD FC PA.

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