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Article

Evaluation of Coinfection and Superinfection Impact on Chikungunya and Mayaro Virus's Replication in *Aedes aegypti*

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Abstract: The co-occurrence of different arboviruses, through (re)emergence episodes in a particular region, often exacerbates public health crises associated with these pathogens. Considering the Brazilian epidemiological panorama of arboviruses cocirculation, such as Dengue (DENV), Zika (ZIKV), and Chikungunya (CHIKV) viruses, the imminent emergence of Mayaro virus (MAYV) in urban areas, and the presence of susceptible human hosts, we looked into the coinfection and superinfection impact of CHIKV and MAYV replication in *Aedes aegypti*. Mosquitoes were coinfecting and superinfected with CHIKV and MAYV by artificial blood-feeding, and infection (IR), dissemination (DR), transmission (TR) rates, and viral load were evaluated by qRT-PCR. Our findings suggest that, *Ae. aegypti* can simultaneously transmit CHIKV and MAYV in coinfection scenarios. However, in superinfection assays, the initial infection (either CHIKV or MAYV) adversely affected the transmission capacity of the subsequently acquired virus, indicating replication interference between the viruses. These results have important implications for public health management actions in the country, emphasizing complex arbovirus transmission scenarios and the associated epidemiological risks.

Keywords: *Aedes aegypti*; *Alphavirus*; cocirculation

1. Introduction

Episodes of emergency and re-emergence of arthropod-borne viruses (arboviruses) are associated with the increasing contact among viruses, vectors, and human hosts [1]. This context is supported by factors such as globalization, anthropogenic changes in biomes, climate change, geographic spread of potential disease vectors, and unsanitary conditions in urban areas [2,3]. As a result, the high density of the vector population, the presence of susceptible hosts, and favorable environmental conditions result on the successful circulation, maintenance, and dissemination of multiple arboviruses in the environment [1–3]. In Brazil, the cocirculation of Dengue (DENV), Zika (ZIKV), and Chikungunya (CHIKV) viruses culminated in the triple epidemic registered in 2015–2016 [4]. Since then, these three viruses are found in urban areas, having humans as vertebrate hosts and *Aedes aegypti* as the main mosquito vector [5]. The cocirculation of arboviruses is influenced by similar biological, ecological, and socioeconomic factors, leading to an epidemiological synergy of seasonality and occurrence rates [2,6–8]. Recently, Mayaro virus (MAYV), an endemic arbovirus widely found in Central America and the Amazon region, has drawn attention due to its ability to invade urban areas and cause outbreaks [9–11], with cases reported in the North, Midwest, Southeast, and Northeast regions of Brazil [12].

One of the main factors for an arbovirus, such as MAYV, to establish itself in an urban environment is the presence of competent mosquito species for the virus [13–15]. In the sylvatic cycle, MAYV has non-human primates and other mammals as vertebrate hosts and *Haemagogus* spp. mosquitoes as vectors [9,11]. On the other hand, entomological surveillance studies conducted in the country have detected *Ae. aegypti* naturally infected with MAYV in the Brazilian states of Mato Grosso [16] and Goiás [17]. Thus, the regular notification of MAYV cases near the main tropical cities, *Ae. aegypti* as a main urban vector, and the presence of susceptible human hosts result in a higher potential for establishing MAYV in an urban cycle [18].

From the standpoint of the vector biology, it is important to understand the dynamics between different viruses in the same individual mosquito. This phenomenon includes the coinfection (simultaneous infection of the mosquito by the viruses in a single blood meal) and superinfection (sequential infection of the mosquito by the viruses, involving more than one blood meal) mechanisms [19,20]. Both coinfection and superinfection can result in different scenarios: virus amplification; virus inhibition; competition between viruses; and neutrality [2,21]. In nature, superinfection is more likely to occur when compared to coinfection, as the mosquito becomes infected sequentially by performing a blood meal on several viremic hosts instead of a single meal on a coinfecting viremic host [2,22,23]. Also, superinfection is commonly related to viral interference, referring to the inability of a virus to replicate in an organism or cell that is previously infected by another virus [24,25], which was already demonstrated between Yellow Fever virus (YFV) and DENV-2 [26], MAYV and ZIKV [21], and CHIKV and MAYV [22].

Therefore, in a setting of arboviruses cocirculation in Brazil and a high density of *Ae. aegypti* mosquitoes, we investigated how coinfection and superinfection impact the virus dynamics between two alphaviruses of a high epidemiological importance, CHIKV and MAYV, using an *Ae. aegypti* colony. The study consisted of blood-meal experiments, drawing different methods for coinfection and superinfection assays, and comparative analyses for the consequent scenarios.

2. Materials and Methods

2.1. Viral Stock

CHIKV (CHIKV BRPE408/2016) and MAYV (MAYV/BR/Sinop/H307/2015) (MH513597) stocks were gently provided by Dr. Marli Tenório (Department of Virology, LAVITE, Aggeu Magalhães Institute) and Dr. Roberta Bronzoni (Federal University of Mato Grosso, UFMT-Sinop), respectively. Viral stocks and viral titration were performed using VERO cells and plaque assays to determine plaque-forming units per mL (PFU/mL), using the protocol described in Krokovsky et al. [15].

2.2. *Aedes aegypti* Colony

The *Ae. aegypti* colony (RecL) used in the present study was derived from a natural population collected in Recife, Pernambuco, Brazil [27]. Mosquitoes were maintained under standard conditions of 26 ± 2 °C, 65–85% relative humidity, and photoperiod of 12/12 light-dark, until adults were obtained to form the groups used in each assay.

2.3. *Aedes aegypti* Artificial Blood-Feeding

Single infection, coinfection and superinfection assays were performed with CHIKV and MAYV. These experiments consisted of two independent artificial blood meals each one, in a BSL2 infectory (Biosafety Level 2). Seven to ten-day-old mosquitoes from the RecL colony were separated into different plastic cages containing 100 females and 20 males placed and maintained in containment cages. Twenty-four hours before blood feeding, mosquitoes were sugar-starved. MAYV and CHIKV were obtained from VERO cells collected 24 h post-inoculation at a 0.1 multiplicity of infection (MOI), while uninfected cultured cells were used in negative control groups.

For the blood meals, cell culture flasks were infected and mixed with defibrinated rabbit blood (1:1) and 3 mM ATP, then presented to the mosquitoes for up to one hour in plastic Petri dishes covered by a triple layer of parafilm paper, as previously described in Guedes et al. [28]. After the

blood feeding, fully engorged females were cold anesthetized, transferred to a new cage, and maintained for 13 days post-exposure (dpe). Infected cell cultures used in each assay were collected to control the viral title offered in the blood meals (Table 1).

Table 1. Average initial viral titer, measured in Plaque Forming Units (PFU), for MAYV and CHIKV used on the single infection, coinfection and superinfection assays.

Group	Virus	1st blood-meal	2nd blood-meal
Single infection- MAYV	MAYV	5,5 x 10 ⁷	-*
Single infection- CHIKV	CHIKV	3,1 x 10 ⁷	-*
Coinfection MAYV + CHIKV	MAYV	5,5 x 10 ⁷	-*
	CHIKV	3,1 x 10 ⁷	-*
Superinfection by MAYV	CHIKV	1,4 x 10 ⁷	-
	MAYV	-	3,1 x 10 ⁷
Superinfection by CHIKV	MAYV	3,5 x 10 ⁷	-
	CHIKV	-	8,5 x 10 ⁶

* non-infectious blood meal.

For single infections by MAYV or CHIKV, mosquitoes were first given an infectious blood meal with the respective virus, followed by a non-infectious blood meal six days later. In the coinfection group, both viruses were present in equal proportions in a single blood meal, followed by a non-infectious blood meal six days later. For the superinfection groups, two separate blood meals with different viruses were administered, spaced six days apart. Specifically, for CHIKV superinfection, the first blood meal contained MAYV, followed by a second blood meal containing CHIKV. Conversely, for MAYV superinfection, the initial blood meal contained CHIKV, followed by a second blood meal containing MAYV (Figure 1).

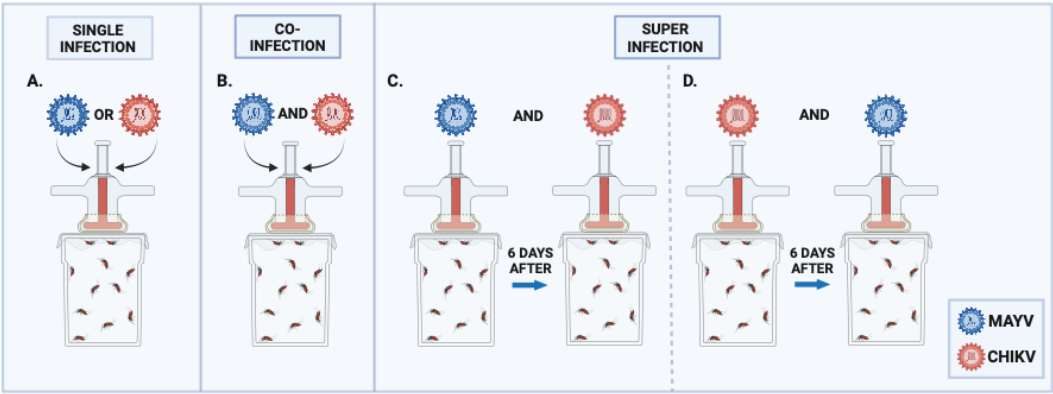


Figure 1. Groups of mosquitoes analyzed in the single infection, coinfection and superinfection experiments: (A) Single infection with MAYV or CHIKV. (B) Coinfection with MAYV and CHIKV. (C) Superinfection with CHIKV. (D) Superinfection with MAYV.

2.4. Sample Collection

The single infection, coinfection and superinfection experiments were carried out in duplicate assays. This resulted in a total number of samples for each group (midguts, carcasses, and saliva) of

n=10 (5 for each replicate) for the single infected and negative control groups and n=30 samples (15 for each replicate) for the coinfecting and superinfected groups at each time-point.

Midguts and carcasses were dissected from female mosquitoes collected at 6th and 12th dpe and cold anesthetized at -20 °C, washed individually in 70% alcohol and distilled water, and deposited in 1x PBS buffer (phosphate buffered saline) (Gibco). Tissues were dissected in a Petri dish containing 1x PBS with the aid of entomological pins and tweezers and visualized with a binocular stereomicroscope (LABOMED). Samples were individually transferred to a 1.5 ml DNase/RNase-free microtube containing 300 µl of mosquito diluent and stored at -80 °C until processing.

For saliva collection, female mosquitoes were collected at 7th and 13th dpe and cold anesthetized (-20 °C). The legs and wings were removed to stimulate salivation and the proboscis was immersed in 100 µl tips with 10 µl of 1x PBS buffer for 30 minutes. After the forced salivation, described by Gloria-Soria et al. with modifications [29], the 1x PBS buffer and the saliva were expelled into 1.5 ml DNase/RNase-free microtubes containing 10 µl of 1x PBS buffer and stored at -80 °C until qRT-PCR assay.

2.5. RNA Isolation and qRT-PCR

Collected tissues were individually homogenized as previously described in Barbosa et al. [30], and RNA extraction was performed using the TRIzol® (Invitrogen, Carlsbad, CA, USA) protocol, described in Guedes et al. [28]. The RNA extracted from the midguts, carcasses, and saliva samples were used as templates in qRT-PCR reactions carried out using the QuantiNova Kit Probe RT-PCR Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) in a final volume of 10 µl (3.5 µl of RNA) and final concentrations of 1× for QuantiNova Probe RT-PCR Master Mix, QuantiNova ROX, and QuantiNova RT Mix, and 800 nM of CHIKV primers, 1200 nM of MAYV primers, 200 nM of CHIKV probe, and 300 nM of MAYV probe. The sequence of primers and probes for CHIKV and MAYV were previously described by Lanciotti et al. [31] and Naveca et al. [32], respectively.

Reactions were performed using a QuantStudio 5 System (Applied Biosystems, Norwalk, CT, USA) under the following conditions: 45 °C for 15 minutes, 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 5 seconds, and 60 °C for 45 seconds. Samples were analyzed in duplicates using negative reaction control, which corresponds to all reagents except for RNA template, negative RNA extraction control, and positive controls corresponding to a standard curve for each virus (MAYV and CHIKV), synthesized as described in Krovovsky et al. [33]. qRT-PCR results were analyzed using QuantStudio Design and Analysis Software v. 1.3.1 with automatic threshold and baseline. Samples with Cq (Cycle quantification) values ≤ 38.5 for both MAYV and CHIKV were considered positives.

2.6. Data Analyzes

The infection rate (IR = positive midguts/midguts analyzed) and dissemination rate (DR = positive carcasses/positive midguts) were calculated at 6 and 12 dpe, meanwhile the transmission rate (TR = positive saliva/saliva analyzed) was evaluated at 7 and 13 dpe. Statistical analyses were performed using GraphPad Prism 8 software (Graph Pad, San Diego, CA, USA). The Wilcoxon test was used to compare the viral load (RNA copy number/ml) between the different groups and time-points. Analyzed results were considered significant when the p-value < 0.05.

3. Results

3.1. Single Infection and Coinfection Assays

The IR for MAYV-single infected mosquitoes was 100% and 90% on the 6th and 12th dpe, respectively (Figure 2A), while the DR was 100% for both time-points (Figure 2C). Regarding the analysis of RNA copy number/ml, it ranged from $1,4 \times 10^4$ to $7,7 \times 10^{10}$ on 6th dpe and $1,1 \times 10^5$ to 1×10^{11} on 12th dpe in the midguts samples (Figure 2B), while ranges from $5,9 \times 10^6$ to $2,2 \times 10^{11}$ on 6th dpe and $1,1 \times 10^7$ to $5,6 \times 10^{12}$ on 12th dpe (Figure 2D) were identified for carcasses (Figure 2D). CHIKV-single infected mosquitoes presented a 100% IR and DR for both time-points (Figure 3A and 3C). The RNA copy number/ml ranged from $1,5 \times 10^{10}$ to $8,3 \times 10^{12}$ on 6th dpe and $2,4 \times 10^{10}$ to $3,6 \times$

10^{12} on 12th dpe in the midguts samples (Figure 3B), and for carcasses, ranges from $1,2 \times 10^{12}$ to $1,8 \times 10^{14}$ on 6th dpe and $1,4 \times 10^{12}$ to $3,5 \times 10^{13}$ on 12th dpe were observed (Figure 3D).

Concerning the coinfecting group, CHIKV presented a higher IR (96.7% on 6th dpe and 93.3% on 12th dpe) (Figure 3A) than MAYV (93.2% on both 6 and 12 dpe) (Figure 3A), as well as a greater viral load (range from $1,2 \times 10^7$ to $2,7 \times 10^{12}$ RNA copy number/ml on 6th dpe and $3,7 \times 10^9$ to $4,4 \times 10^{12}$ on 12th dpe) than MAYV (range from $2,5 \times 10^6$ to $1,1 \times 10^{10}$ RNA copy number/ml on 6th dpe and 1×10^7 to $4,2 \times 10^{10}$ on 12th dpe) (Figures 3B and 2B). Furthermore, both viruses revealed a 100% DR for both time-points analyzed (Figures 2C and 3C); however, CHIKV displayed a higher viral load (range from $3,3 \times 10^7$ to $2,3 \times 10^{13}$ RNA copy number/ml on 6th dpe and $1,7 \times 10^8$ to $2,7 \times 10^{13}$ on 12th dpe) than MAYV (range from $1,5 \times 10^5$ to $8,9 \times 10^{10}$ RNA copy number/ml on 6th dpe and $1,9 \times 10^5$ to 3×10^{11} on 12th dpe) (Figures 2D and 3D).

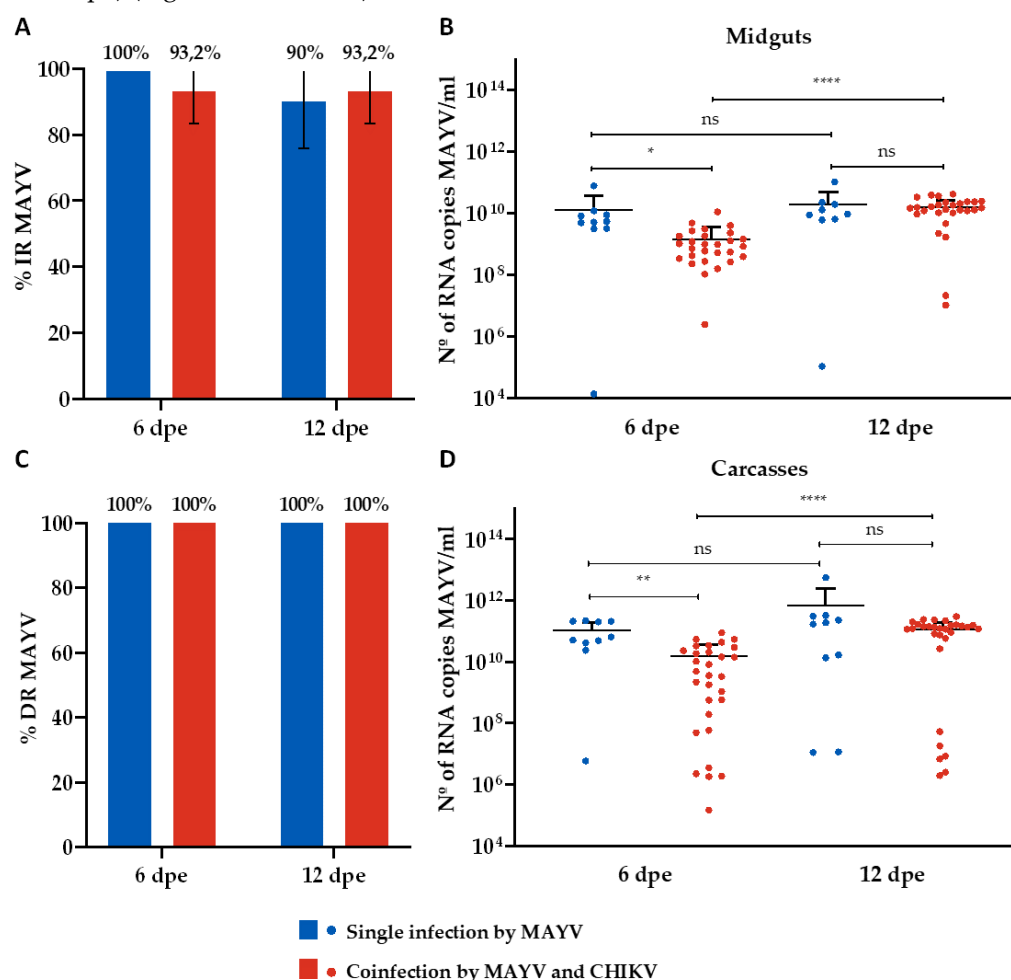


Figure 2. Infection and Dissemination rates (%) for MAYV and number of MAYV RNA copies/ml in the single infection and coinfection assays. (A) Infection rate (IR); (B) Viral RNA copy number/ml in positive midguts; (C) Dissemination rate (DR); (D) Viral RNA copy number/ml in positive carcasses. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ samples of each tissue (midgut and carcass) for MAYV single-infected group and $n=30$ for MAYV and CHIKV coinfecting group.

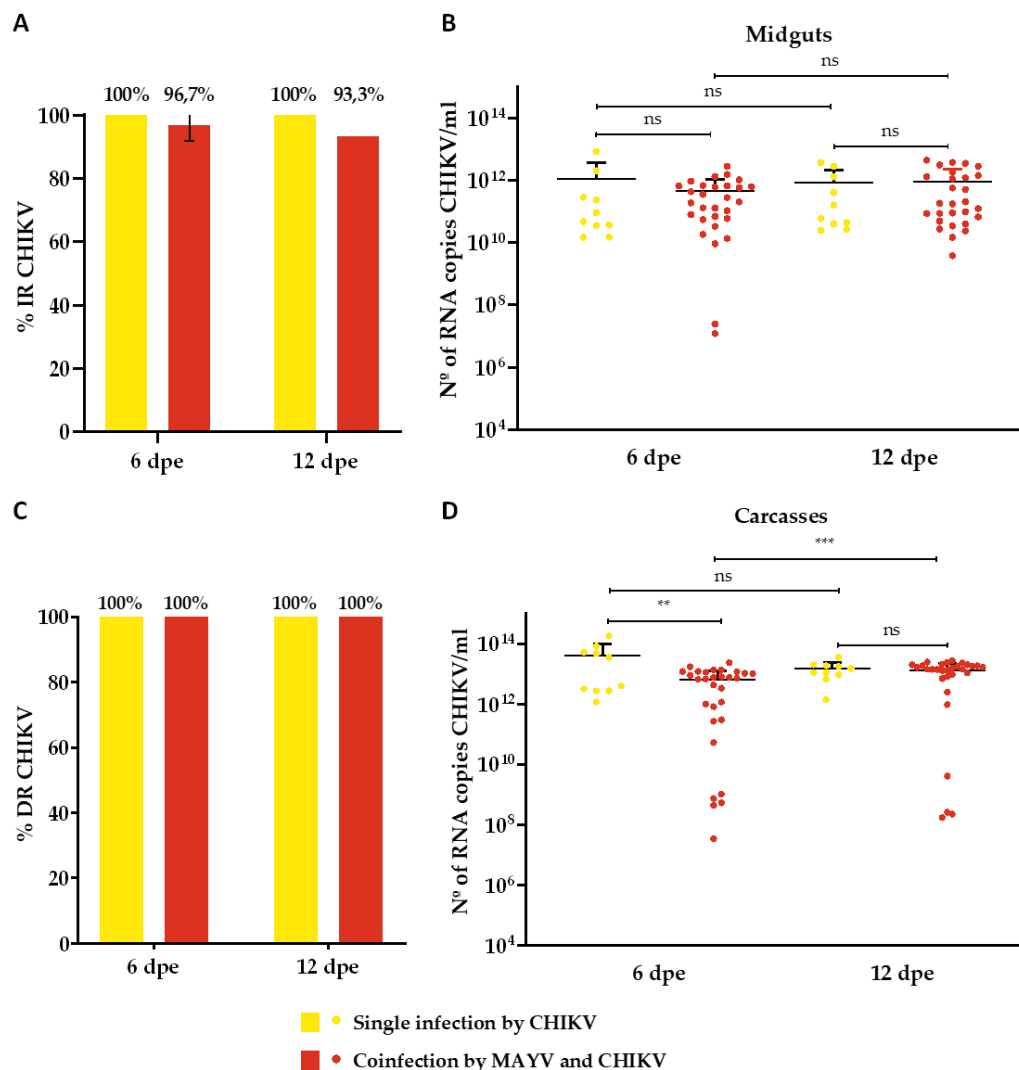


Figure 3. Infection and Dissemination rates (%) for CHIKV and number of CHIKV RNA copies/ml in the single infection and coinfection assays. (A) Infection rate (IR); (B) Viral RNA copy number/ml in positive midguts; (C) Dissemination rate (DR); (D) Viral RNA copy number/ml in positive carcasses. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ samples of each tissue (midgut and carcass) for CHIKV single-infected group and $n=30$ for MAYV and CHIKV coinfecting group.

Regarding TR, mosquitoes that were single-infected with MAYV showed positivity rates of 30% and 10% on the 7th and 13th dpe, respectively (Figure 4A). The RNA copy numbers/ml ranged from $2,8 \times 10^5$ to $2,6 \times 10^7$ on the 7th dpe and were $8,6 \times 10^4$ per ml on the 13th dpe (Figure 4B). Conversely, mosquitoes that were single-infected with CHIKV showed no positivity (Figures 4C and 4D). For the coinfecting mosquitoes, a TR of 16.7% was observed for MAYV at both time points (Figure 4A), with RNA copy numbers/ml ranging from $3,9 \times 10^4$ to 2×10^6 on the 7th dpe and from $4,9 \times 10^4$ to 3×10^8 on the 13th dpe (Figure 4B). For CHIKV, a TR of 7.2% was noted on the 7th dpe (Figure 4C), with RNA copy numbers/ml ranging from 1.7×10^9 to $1,5 \times 10^{10}$ (Figure 4D).

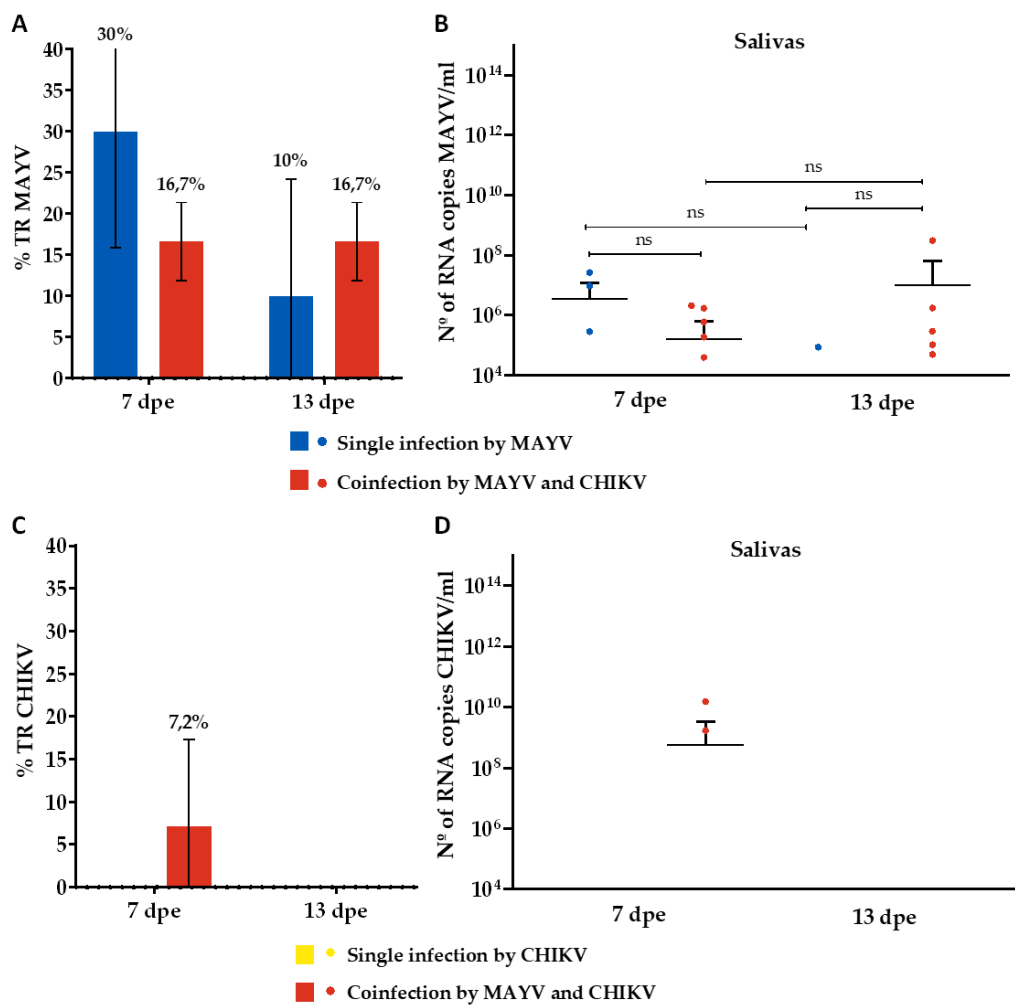


Figure 4. Transmission rate (%) for MAYV and CHIKV and number of MAYV and CHIKV RNA copies/ml in the coinfection assay. (A) Transmission rate (TR) for MAYV; (B) MAYV RNA copy number/ml in positive saliva; (C) Transmission rate (TR) for CHIKV; (D) CHIKV RNA copy number/ml in positive saliva. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ saliva for MAYV and CHIKV single-infected groups and $n=30$ for MAYV and CHIKV coinfecting group.

3.2. Superinfection Assay

CHIKV-superinfected mosquitoes presented a 70% and 93.3% IR on 6th dpe and on 12th dpe, respectively, for MAYV (Figure 5A), and an 83.3% IR on 12th dpe for CHIKV (Figure 6A). Furthermore, it revealed an 83.3% and 93.1% DR on 6th dpe and on 12th dpe, respectively, for MAYV (Figure 5C), and an 85.7% DR on 12th dpe for CHIKV (Figure 6C). Regarding the analysis of MAYV RNA copy number/ml, ranges from $5,6 \times 10^6$ to 1×10^{12} on 6th dpe and from 1×10^7 to 2×10^{12} on 12th dpe were found for midguts (Figure 5B), while ranges from 1×10^7 to $2,5 \times 10^{12}$ on 6th dpe and 14×10^8 to $5,9 \times 10^{13}$ were identified for carcasses (Figure 5D). Analysis of CHIKV RNA copy numbers/ml showed ranges from $3,8 \times 10^8$ to $1,4 \times 10^{12}$ on 12th dpe for midguts (Figure 6B), and ranges from $1,3 \times 10^7$ to $1,3 \times 10^{13}$ on 12th dpe were identified for carcasses (Figure 6D).

For mosquitoes superinfected with MAYV, the infection rates (IR) for CHIKV were 73.3% on the 6th dpe and 76.7% on the 12th dpe (Figure 6A). For MAYV, the IR was 7.3% on the 12th dpe (Figure 5A). The DR for CHIKV was 95.5% on the 6th dpe and 100% on the 12th dpe (Figure 6C), while the DR for MAYV was 71.4% on the 12th dpe (Figure 5C). Analysis of CHIKV RNA copy numbers/ml showed ranges from $2,4 \times 10^6$ to $5,4 \times 10^{11}$ on the 6th dpe and from 4×10^5 to $3,1 \times 10^{12}$ on the 12th dpe for midguts (Figure 6B). For carcasses, the ranges were from $3,6 \times 10^6$ to 9×10^{12} on the 6th dpe and

from $3,3 \times 10^7$ to $5,7 \times 10^{12}$ on the 12th dpe (Figure 6D). Regarding MAYV RNA copy numbers/ml, ranges from 4×10^7 to $2,3 \times 10^{12}$ were observed on the 12th dpe for midguts (Figure 5B) and from $1,7 \times 10^8$ to $2,7 \times 10^{13}$ for carcasses (Figure 5D).

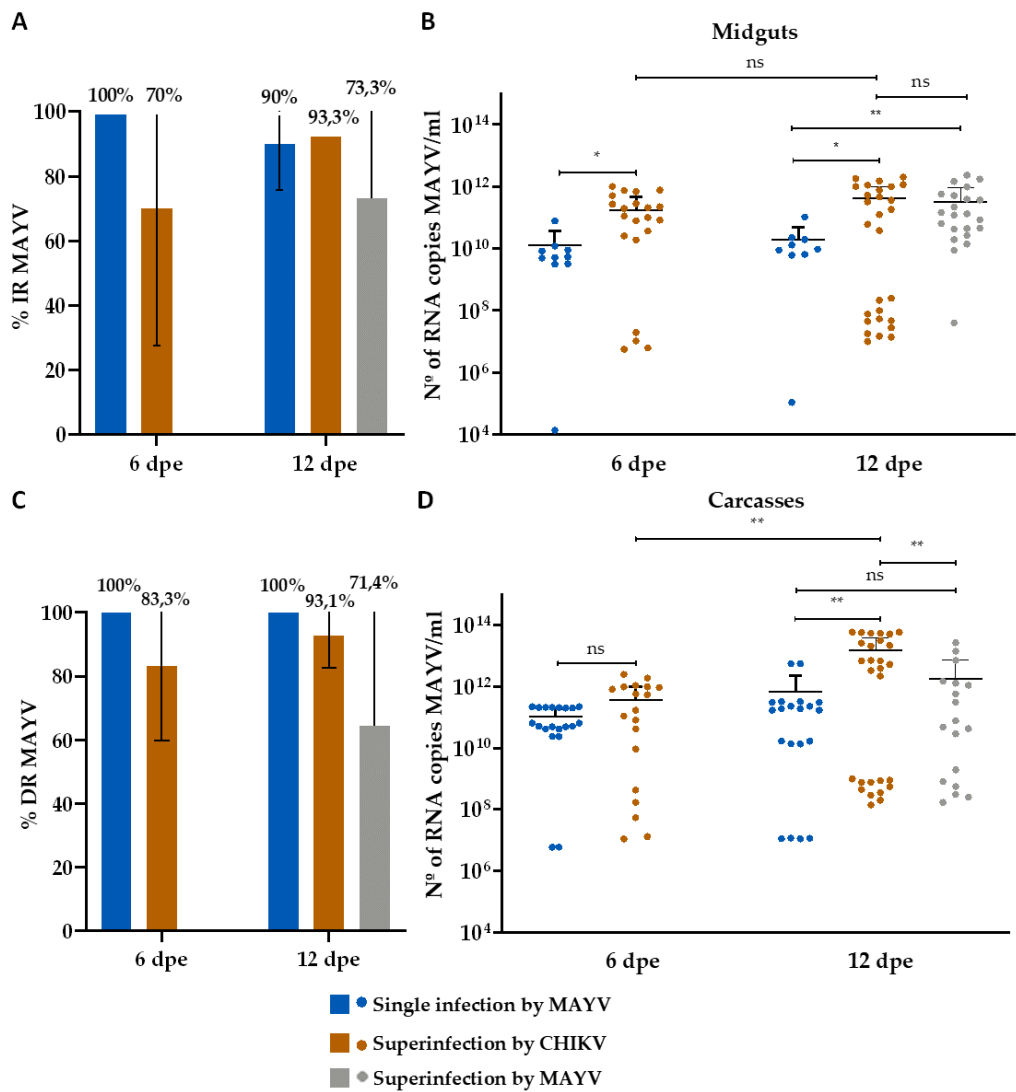


Figure 5. Infection and Dissemination rates (%) for MAYV and number of MAYV RNA copies/ml in the superinfection assay. (A) Infection rate (IR); (B) Viral RNA copy number/ml in positive midguts; (C) Dissemination rate (DR); (D) Viral RNA copy number/ml in positive carcasses. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ samples of each tissue (midgut and carcass) for MAYV single-infected group and $n=30$ for MAYV or CHIKV superinfected groups.

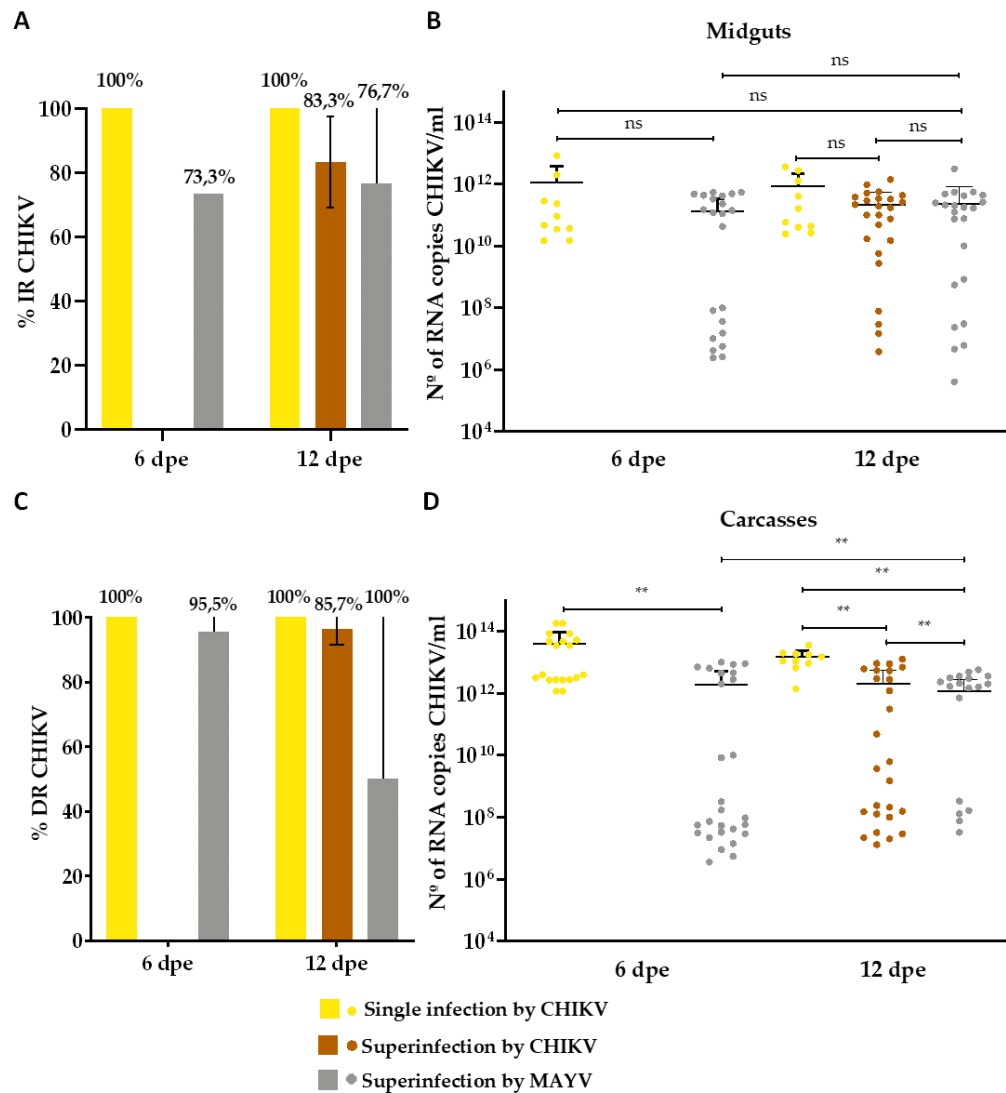


Figure 6. Infection and Dissemination rates (%) for CHIKV and number of CHIKV RNA copies/ml in the superinfection assay. **(A)** Infection rate (IR); **(B)** Viral RNA copy number/ml in positive midguts; **(C)** Dissemination rate (DR); **(D)** Viral RNA copy number/ml in positive carcasses. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ samples of each tissue (midgut and carcass) for CHIKV single-infected group and $n=30$ for MAYV or CHIKV superinfected groups.

Concerning the TR in the CHIKV-superinfection assay, MAYV was detected in 6.7% of the saliva on the 7th dpe (Figure 7A) (range from $2,5 \times 10^9$ to $1,8 \times 10^{11}$ RNA copy number/ml) (Figure 7B), while MAYV-superinfected mosquitoes showed a 3.3% positivity for CHIKV on 13th dpe (Figure 7C) ($3,9 \times 10^{10}$ RNA copy number/ml) (Figure 7D).

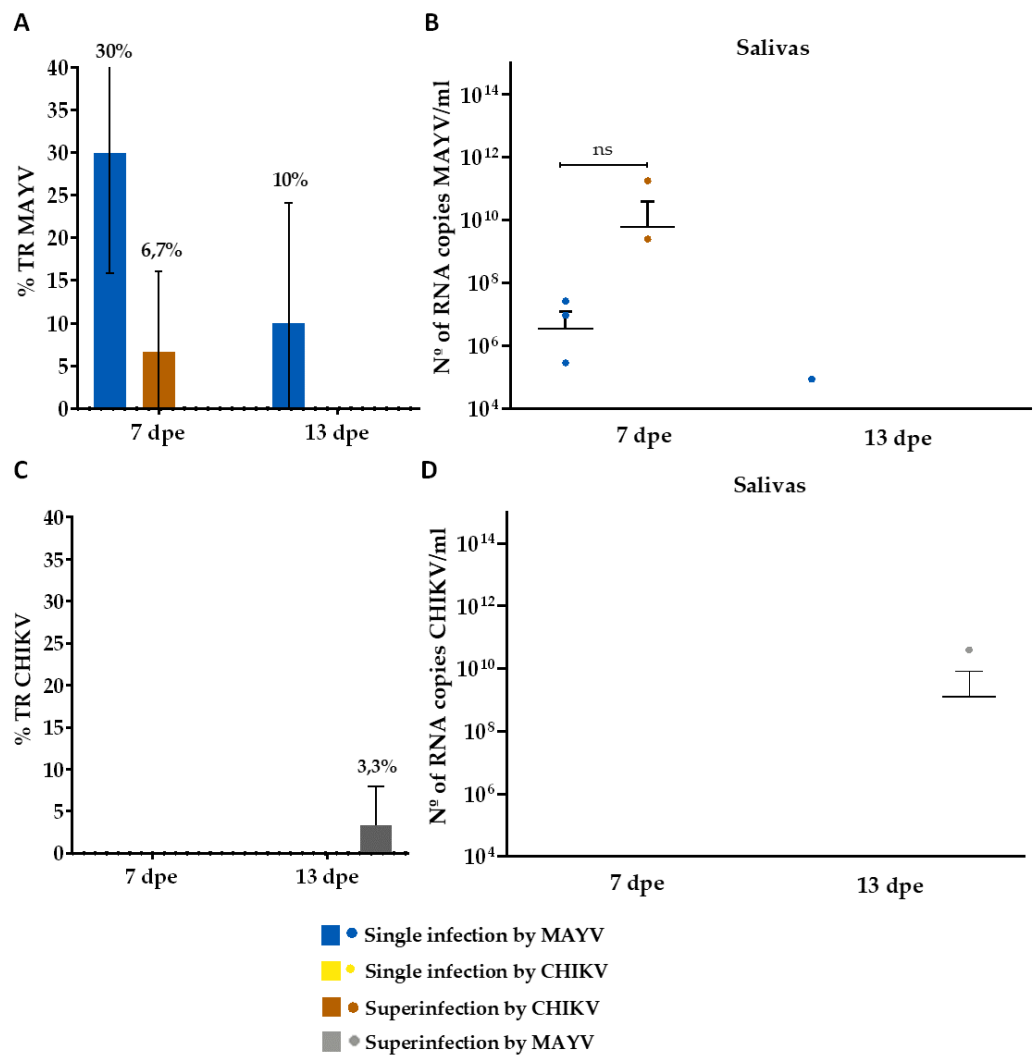


Figure 7. Transmission rate (%) for MAYV and CHIKV and number of MAYV and CHIKV RNA copies/ml in the superinfection assay. (A) Transmission rate (TR) for MAYV; (B) MAYV RNA copy number/ml in positive saliva; (C) Transmission rate (TR) for CHIKV; (D) CHIKV RNA copy number/ml in positive saliva. Dpe- days post-exposure; * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$, **** $p \leq 0,0001$, ns - non-significant. It was analyzed $n=10$ saliva for MAYV and CHIKV single-infected groups and $n=30$ for MAYV or CHIKV superinfected groups.

4. Discussion

The current epidemiological landscape on a global scale has been characterized by numerous cases of arbovirus (re)emergence, accompanied by their rapid geographical dissemination [1]. This phenomenon has prompted the simultaneous circulation of diverse pathogens within particular regions [34]. In Brazil, alongside DENV, ZIKV, CHIKV, and YFV, Oropouche virus (OROV) has recently emerged as an increasingly notified virus, surpassing four thousand cases [35]. In this context, considering the potential emergence of MAYV in urban regions of Brazil, a country with the highest reported cases of MAYV human infections [9–12,18], and the favorable conditions for the simultaneous circulation of arboviruses- including the presence of competent vectors and susceptible vertebrate hosts [5]- it becomes imperative to comprehend the relationship among various viruses within the same vector [22]. This area of investigation remains full of gaps in the scientific literature, underscoring its critical significance [21]. Confronted with this scenario, we investigated the coinfection and superinfection consequences on the replication of CHIKV and MAYV in *Ae. aegypti* mosquitoes.

In coinfection scenarios, both CHIKV and MAYV replicate similarly to single infections, indicating minimal interference in the transmissibility of the viruses and, consequently, in vector competence. Previous studies of vector competence involving *Ae. aegypti* have shown this mosquito has the ability to become infected and transmit multiple viruses during a single blood meal, such as combinations of DENV, ZIKV, and CHIKV [36,37], ZIKV-MAYV [21], and, as observed in this study, CHIKV-MAYV [22], supporting the hypothesis that coinfection event does not significantly interfere with the amplification of the viruses [2,37,38]. On the other hand, we noted interference in the transmission of the viruses during the second blood meal in the superinfection experiments by CHIKV and MAYV. This shows the occurrence of a potential inhibition of viral replication. The group superinfected with CHIKV displayed a predominance of MAYV (first blood meal), as only MAYV was detected in saliva collected from the 7th dpe. Conversely, the group superinfected with MAYV displayed a prevalence of CHIKV (first blood meal), as supported by TR results in which only CHIKV was detected in samples collected on the 13th dpe. Previous studies have also found similar results. Kantor et al. [22] found interference in the transmission between MAYV and CHIKV in *Ae. aegypti* mosquitoes superinfected by CHIKV, similar to our findings. However, they did not find interference in the reverse scenario (mosquitoes superinfected by MAYV). In contrast, our study identified interference in mosquitoes superinfected by MAYV as well. Additionally, Abrao & Da Fonseca [26] reported, through in vitro superinfection studies, a reduced viral replication of Yellow Fever virus (YFV) in C6/36 cells previously infected with DENV-2 when compared to single infections by YFV [26].

Previous studies have discussed factors that may be associated with interference, inhibition, or even exclusion of viral replication in a superinfection scenario [26]. One key factor is the activation of the vector host's immune system by the primary viral infection, which can make it difficult for a subsequently acquired arbovirus to replicate [2,26]. Additionally, other factors could be involved, such as the vector's microbiome competition, a decrease in cellular resources required for the replication of the secondarily acquired virus, reduced binding of the virion to cell surface receptors during the second infection, and inhibition of the nucleocapsid uncoating related to the second infection [25,39,40]. However, this phenomenon remains somewhat ambiguous, as certain studies have indicated no interference in cases of superinfection. For instance, Kuwata et al. [41] demonstrated in vitro that a pre-existing infection with CxFV (an insect-specific flavivirus initially isolated from *Culex* spp.) did not interfere with Japanese Encephalitis virus (JEV) or DENV infection at the cellular (CTR cells) level. Similarly, Kent et al. [42] reported that prior exposure to CxFV had no noticeable effect on West Nile virus (WNV) replication in experiments conducted with C6/36 cells.

The potential for viral interference in superinfection scenarios has been extensively investigated, particularly in the context of insect-specific viruses (ISVs) [24]. These viruses can replicate exclusively in insect cells and can directly impact the infection, dissemination, and transmission of arboviruses, as demonstrated between Nhimirim virus (NHUV) and WNV in *Culex quinquefasciatus* [43], and Eilat virus (EILV) and CHIKV in *Ae. aegypti* [40]. These studies support the exploration of innovative methods for vector control [22,24,39,41]. From a different perspective, viral interference between mammalian pathogenic arboviruses may, to some extent, provide explanations for the infrequent or nonexistent occurrence of certain viruses in regions with a high prevalence of others [24,26]. Abrao & Da Fonseca [26] previously proposed this concept to account for the limited occurrence of YFV in areas with substantial DENV circulation. However, this hypothesis remains speculative, as it requires not only in vitro analyses but also in vivo and field validations for confirmation. Regarding our results, we observed interference in both pathways MAYV-superinfection and CHIKV-superinfection, which allows us to discuss a possible variable that contributes to the absence of MAYV emergence in Brazilian urban areas, characterized by a high circulation of CHIKV and a high density of *Ae. aegypti* [5], which, in turn, demonstrated to be competent for MAYV [15,44]. Similarly, the interference caused by an initial MAYV infection in mosquitoes might contribute to the comparatively low detection rates of CHIKV in the northern regions of Brazil [45]. This phenomenon is compounded by the fact that CHIKV has already adapted to urban environments, undergoing

direct amplification cycles in humans [46], and only emerged in Brazil in 2014 [47], whereas MAYV has been circulating in the northern region since the 20th century [48].

It is important to note potential biases associated with the forced salivation technique, used in the present study, such as its non-standardization, even though it is considered the gold method for assessing transmission rates in vector competence studies without resorting to an animal model, and the non-quantification of saliva expulsion [49]. Given this context, it is crucial to conduct vector competence studies using animal models. Such studies should observe histopathologic effects and quantify viral presence in various tissues, as exemplified by Krokovsky et al. [15]. In our study, we observed low numbers of infected saliva across all tested groups, which implies the existence of a robust barrier within the mosquito's salivary gland against CHIKV and MAYV, despite a viral infection disseminated in the organism [38,50,51]. Low TR have also been observed in prior studies involving CHIKV and MAYV within *Ae. aegypti*. For instance, Göertz et al. [38] reported a TR of 21.2% at 14 dpe for mosquitoes single infected by CHIKV, using initial titers with log 10⁷ TCID50/ml. In the same study, the TR was 14.6% for CHIKV in mosquitoes coinfecting with ZIKV (72.9%), with an initial titer of log 10⁷ TCID50/ml for both viruses [38]. Another study by Wiggins et al. [52] found TR of 10% and 23% at 6 and 12 dpe, respectively, for mosquitoes single infected by MAYV, using an initial viral titer with a log of 10¹⁰ PFU/ml.

Our findings indicate that in a coinfection scenario, both MAYV and CHIKV can replicate in *Ae. aegypti* without adversely affecting viral replication and, consequently, vector competence for both viruses. However, superinfection events by MAYV or CHIKV can disrupt the transmission of the secondary acquired virus. This highlights a notable epidemiological concern, particularly in Brazilian urban areas where multiple viruses, including DENV, ZIKV, CHIKV, YFV, and OROV, circulate, underscoring the critical importance of multiplex diagnostic methods for simultaneous detection of these co-circulating viruses. Therefore, these results bear significant consequences for shaping public health strategies in the region, emphasizing the intricate nature of arbovirus transmission scenarios and the related epidemiological risks.

5. Conclusions

Our study highlights the capability of MAYV and CHIKV to replicate in *Ae. aegypti* during coinfection scenarios, however, with observed interference in viral transmission during superinfection by MAYV or CHIKV events. Therefore, we underscore the importance of considering coinfection and superinfection dynamics in studies related to virus-vector interactions, entomological surveillance programs, and risk assessments in regions susceptible to arbovirus epidemics. The implications of coinfection and superinfection within the fields of epidemiology, pathogenesis, and viral evolution, both in mammalian hosts and mosquito vectors, remain underexplored. Future studies could delve into investigating the impact of these phenomena on the diversity of the viral genome and subsequent changes in the pathogenicity of diseases associated with these arbovirus infections.

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