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# Dengue, Yellow Fever, Zika and Chikungunya epidemic arboviruses in Brazil: ultrastructural aspects.

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## ABSTRACT

The impact of the cocirculation of several arboviruses in Brazil is still little known. As in the case of reinfection by different serotypes of DENV, the interaction of arboviruses (DENV serotypes 1-4, YF, ZIKV and CHIKV) could theoretically result in more intense viremia or other immunological alterations. In 2015 the epidemic of ZIKV in the Americas provided evidence for features of pathogenicity that had not been previously observed in infections by flaviviruses. ZIKV was shown to be able to cross the placental barrier to cause congenital infections and to be transmitted sexually among humans. I*n vitro* studies by transmission electron microscopy show that part of the ZIKV morphogenesis may occur within viroplasm-like structures never seen in other flaviviruses. Current studies suggest that flaviviruses produce an ensemble of structurally different virions circulating in an organism, collectively contributing to tissue tropism and virus dissemination. In this work, *Aedes albopictus* mosquito cell lineage (C6/36 cells) and Kidney epithelial cells of African green monkey (Vero cells)

were infected with samples of these main circulating arboviruses in Brazil (DENV-1, DENV-2, DENV-3, DENV-4, ZIKV, YF and CHIKV) and ultrastructural studies by transmission electron microscopy were performed.

**Key words:** dengue virus, zika virus, yellow fever virus, chikungunya virus, morphology, morphogenesis

## INTRODUCTION

Currently, the Brazilian Public Health System is going through a challenging scenario when the subject in question is the arboviruses. As not enough dengue virus (DENV) have an hyperendemic profile, it has resulted in the cocirculation of the four DENV serotypes (Nunes et al., 2019), the emergence of Zika virus (ZIKV) (Lowe et al., 2018) and Chikungunya virus (CHIKV) (Souza et al., 2019), and the risk of re-urbanization of Yellow Fever Virus (YFV) (Possas et al., 2018). Vector control remains the sole effective method of prevention since there are no effective vaccines and antivirals for all the arboviruses in question (Lima-Camara, 2016; Medeiros et al., 2018). The perplexity of the dissemination of ZIKV and CHIKV and its impact in Brazil was enough to declare a public health emergency by the Ministry of Health and the World Health Organization, almost two years after the introduction of these viruses in Brazil. In intense mobilization of resources and articulations between states and municipalities to confront the viral circulation, which has taken great proportions (Donalisio et al., 2017).

DENV (consisting of four antigenically different serotypes: DENV-1, DENV-2, DENV-3 and DENV-4), YFV and ZIKV belong to the genus *Flavivirus, Flaviviridae* family. The virus particles are rounded with an approximate diameter of 60 nm, have a lipid bi-layered envelope that comprises the E protein (envelope protein) and the prM protein (precursor membrane protein). It is cleaved into the M protein during exocytosis and thus ensuring the infectivity of the viral particle. The icosahedral nucleocapsid consists of a positive-sense single-stranded ribonucleic acid chain (ssRNA) of ∼10.7 kb and the C protein (capsid protein) presenting a diameter of approximate 30nm. The mechanism of ZIKV replication is not well studied in detail so far. A previous study showed that part of the replicative cycle may occur within a viroplasm-like cell compartment, a structure observed never before in other flaviviruses replication cycles (Barreto-Vieira et al., 2017).

CHIKV belong to the *Alphavirus* genus within the *Togaviridae* family. Alphaviruses are enveloped spherical particles with a diameter of 65–70 nm (Simizu et al., 1984; Cao and Zhang, 2013) and its genome consists of a single-stranded positive-sensed 11.8 kB RNA molecule packaged by the C protein to form the nucleocapsid. This nucleocapsid is surrounded by a host-cell derived lipid bilayer with two inserted transmembrane glycoproteins, E1 and E2 (Jose et al., 2009). The composition of the host-cell derived lipid bilayer strongly resembles the plasma membrane of the infected host cell (Kalvodova et al., 2009).

The morphology and morphogenesis of these four arboviruses currently circulating in Brazil were here analyzed using transmission electron microscopy.

## MATERIALS AND METHODS

### Viruses

*Dengue viruses.* The DENV-1, -2, -3 and DENV-4 samples used were isolated in C6/36 cells culture by the Laboratório de Flavivírus, Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz (Fiocruz) from sera obtained during epidemics in the state of Rio de Janeiro, Brazil in the 2008, 2000, 2008 and 2013, respectively. The samples were previously serotyped by indirect immunofluorescence using DENV specific monoclonal antibodies of DENV-1: 15F3, DENV-2: 3H5, DENV-3: 8A1 and DENV-4: Ascitic fluid Anti Dengue 4, and by Real Time Quantitative PCR (qRT-PCR) using specifics primers. The viral titer were DENV-1:$10^{6.075}$PFU/mL, DENV-2:$10^{6.51}$ PFU/mL, DENV-3:$10^{7.08}$PFU/mL and DENV-4: $10^{8,85}$PFU/mL. The viral stock was stored at -$80^{0}$C.

*Yellow Fever virus.* The YFV sample used was the 74018 isolate belonging to the South American genotype I, isolated from Brazil, corresponding to the distinct the lineage 1D FIOCRUZ 74018/MG/01 (YFV-74018) from a human fatal case in 2001. The isolate has been passaged four times in C6/36 cells culture and viral stock were stored at -80°C, until use. The viral titer was 6,5x$10^{6}$ FFU/mL. The sample was provided by the Laboratório de Mosquitos Transmissores de Hematozoários.

*Zika virus.* The ZIKV sample used was isolated in C6/36 cells from a Brazilian patient blood in 2015 by the Laboratório de Flavivirus, IOC, Fiocruz. The sample was tested by qRT-PCR, using specific primers and the complete genome sequence was deposited in the GenBank under the accession number KX197205. The viral titer was 2,8 x $10^{8}$ PFU/mL and the viral stocks were stored at -80°C.

*Chikungunya virus.* CHIKV BHI-373 sample used was isolated from serum of a Brazilian patient serum in C6/36 cells by the Laboratório de Virologia, Instituto Evandro Chagas, Fiocruz in 2014. The viral titer was 6,2x$10^{3}$ PFU/mL and the viral stocks were stored at -80°C.

### Cells

*Aedes albopictus mosquito cell lineage (C6/36 cells).* The viruses samples (DENV-1, -2, -3, -4, ZIKV, CHIKV and YFV) were Inoculated onto monolayer of C6/36 cell (cultivation bottle 25cm$^2$) with 1 multiplicity of infection (MOI 1) and incubated for 1 hour at 28°C for virus adsorption. The monolayers were maintained in Leibovitz medium (L-15) (Invitrogen) supplemented with 1% nonessential amino acids (Invitrogen), and 5% fetal bovine serum (FBS) (GIBCO), and 1% penicillin/streptomycin (P/S) (Invitrogen) at 28°C. Cytopathic effects (CPE) were investigated at 24, 48 and 72hr post-infection (p.i.) by inverted light microscopy.

*Kidney epithelial cells of African green monkey (Vero cells).* ZIKV and CHIKV samples MOI 1 were inoculated onto monolayers of Vero Cells-CCL-81 that was adsorbed by the cells for 1 hour at 37ºC. After incubation period, the monolayers were maintained with Earle's 199 (Invitrogen) medium supplemented with 5% FBS (GIBCO), 2mML-glutamine and 100U/50µg P/S (Invitrogen), under an atmosphere containing 5% $CO_2$ and incubated at 37°C. CPE was investigated at 24, 48 and 72hr p.i. by inverted light microscopy.

**Cell processing for transmission electron microscopy analysis**

Cells were fixed in 1% glutaraldeyde in sodium cacodylate buffer (0.2 M, pH 7.2) (Electron Microscopy Science), post-fixed in 1% buffered osmium tetroxide (Electron Microscopy Science), dehydrated in acetone (Merck), embedded in epoxy resin (Electron Microscopy Science) and polymerized at 60$^0$C during three days. Ultrathin sections (50-70 nm thick) were obtained from resin blocks. The sections were picked up onto copper grids, stained with uranyl acetate (Electron Microscopy Science) and lead citrate (Electron Microscopy Science), and observed in a Jeol JEM 1011 transmission electron microscope.

## RESULTS

**Flaviviruses:** Dengue virus, Yellow Fever virus and Zika virus

**A. Virus particles morphology** (Figure 1)

Virus particles and replication were observed in all infected cells cultures. The DENV particles a showed spherical shape with an approximate diameter of 60nm and an evident envelope structure (Figures 1A-D). No morphological difference was observed between the particles of the four serotypes. Cells infected with YFV showed viral

particles of a spherical shape with an approximate diameter of 70nm. The envelope structure was as evident as it was in DENV particles (figure 1E). The morphology of ZIKV particles as well as the DENV and YFV is spherical with about 40-50 nm of diameter (Figure 1F).

The particle morphology of the four serotypes of DENV, YFV and ZIKV was similar.

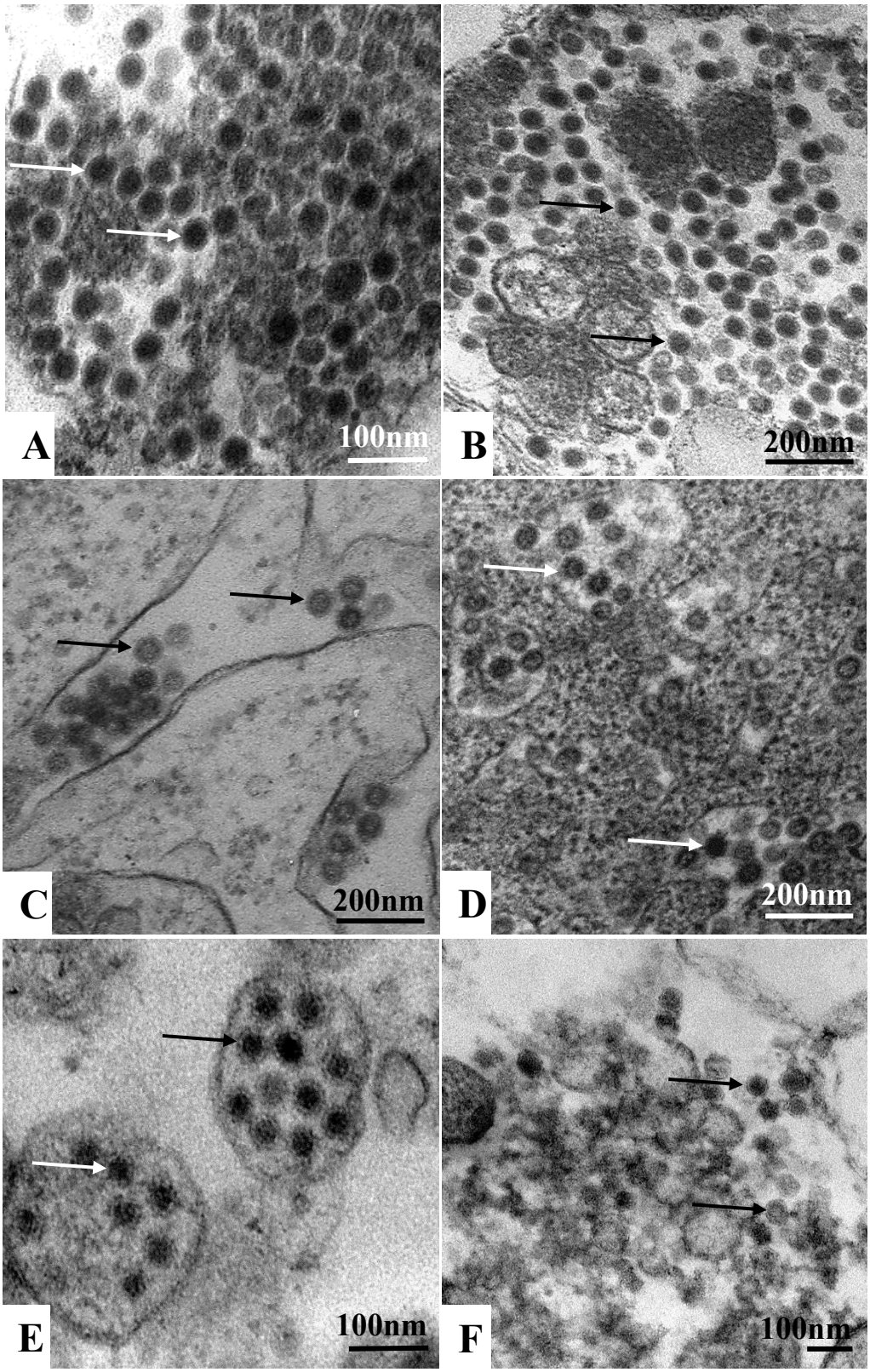
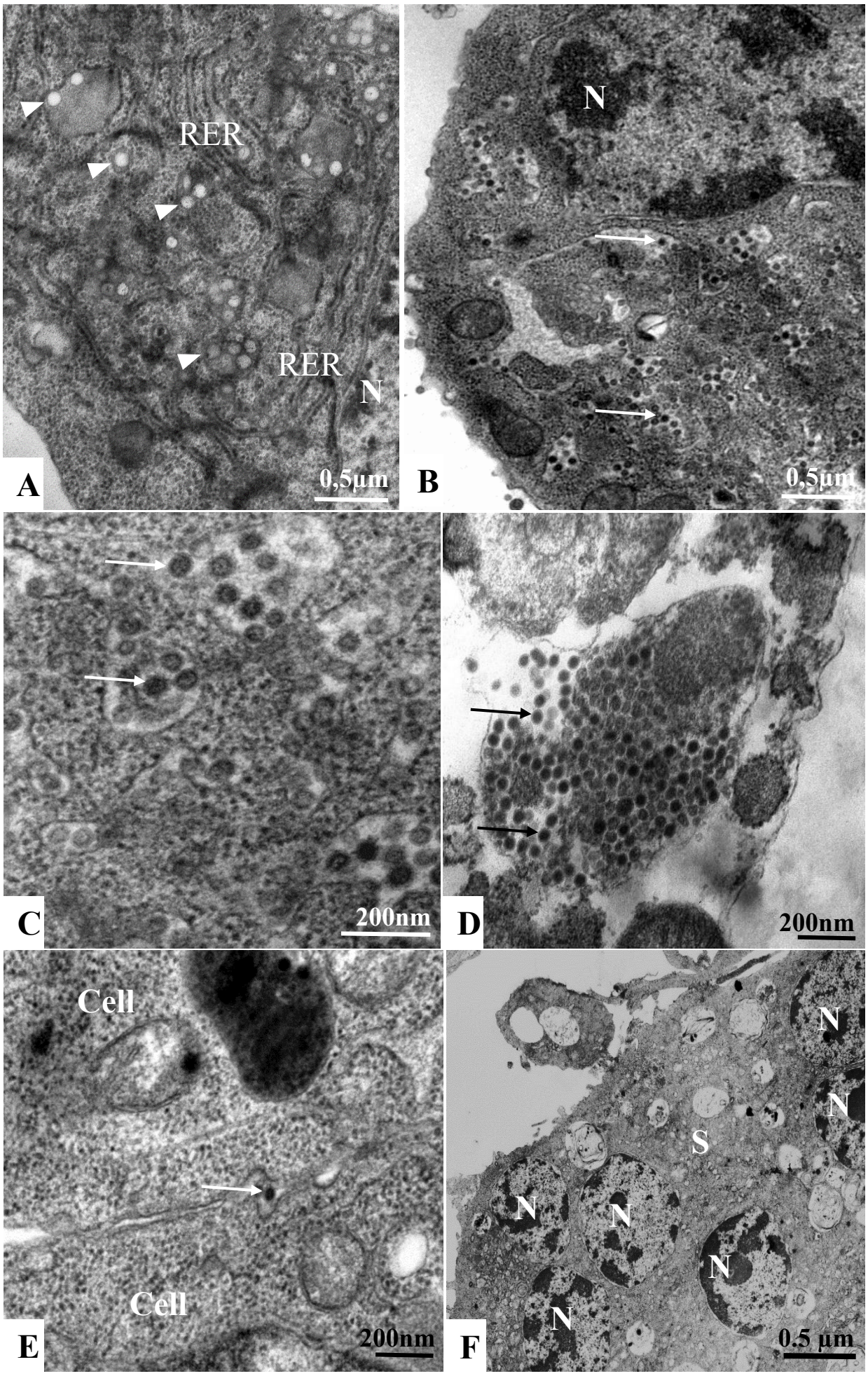



**Figure 1.** Dengue, Yellow Fever and Zika virus particles (arrows) inside cytoplasmic vesicles of C6/36 and Vero cells analyzed by transmission electron microscopy. **A**: Dengue virus serotype 1 (C6/36 cells, 72hr post-infection), **B**: Dengue virus serotype 2 (C6/36 cells, 72 hours post-infection), **C**: Dengue virus serotype 3 (C6/36 cells, 72 hours post-infection), **D**: Dengue virus serotype 4 (C6/36 cells, 72hr post-infection). The measuring of four serotypes of dengue particles was around 60nm in diameter. **E.** Yellow Fever virus particles with an approximate diameter of 70nm (C6/36 cells, 7 days

post-infection), **F**: Zika virus particles with about 40-50 nm of diameter (Vero cell, 48hr post-infection).

**B. Virus morphogenesis** (Figures 2-4)

The internalization of the viral particles in C6/36 cells infected with the DENV-1, -2, -3 and -4 and YFV was observed early inside endocytic vesicles coated with clathrin (Figure 3A). Later, the infected cells presented spherical and tubular structures (Figures 2A, 3B) and, in the sequence, it is possible to observed viral particles during its assembly process (nucleocapsids, Figure 3C), and particles already presenting an envelope inside cisterns of the rough endoplasmic reticulum (Figures 2B, 2C, 3D), in cytoplasmic vesicles (Figures 2D, 3E) and in cell fusion (Figure 2E). The cytophatic effect showed syncytia (Figure 2F). C6/36 and Vero cells infected with ZIKV showed an increased number of ribosomes (Figure 4A), myelin figures, numerous phagosomes (figure 4B) and lysosomes (Figure 4D). Thickening of nuclear membrane and vesicular compartments, measuring around 100 nm in diameter, associated with the rough endoplasmic reticulum were observed (data not shown). Large viroplasm-like compartments (Figures 4C, 4D), localized in the perinuclear area, together with peripheric rough endoplasmic reticulum, mitochondria and microtubules, were verified. ZIKV particles were observed inside lysosomes (Figure 4E), rough endoplasmic reticulum (Figure 4E) and viroplasm-like structures (Figures 4C).


RER
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F

**Figure 2. DENV.** C6/36 cells, 72 hours post-infection, transmission electron microscopy. **A**: Cell infected with dengue virus serotype 1 presenting tubular structures (arrowhead) associated with the rough endoplasmic reticulum (RER), **B**/**C**: Dengue virus serotype 4 particles inside a cytoplasmic vesicles (arrows), **D**: Dengue virus serotype 1 inside a cytoplasmic vesicle (arrows), **E**: Dengue virus serotype 1 (arrow) intermediate the fusion of two cells, **F**: Syncytium (S). Nucleus (N).

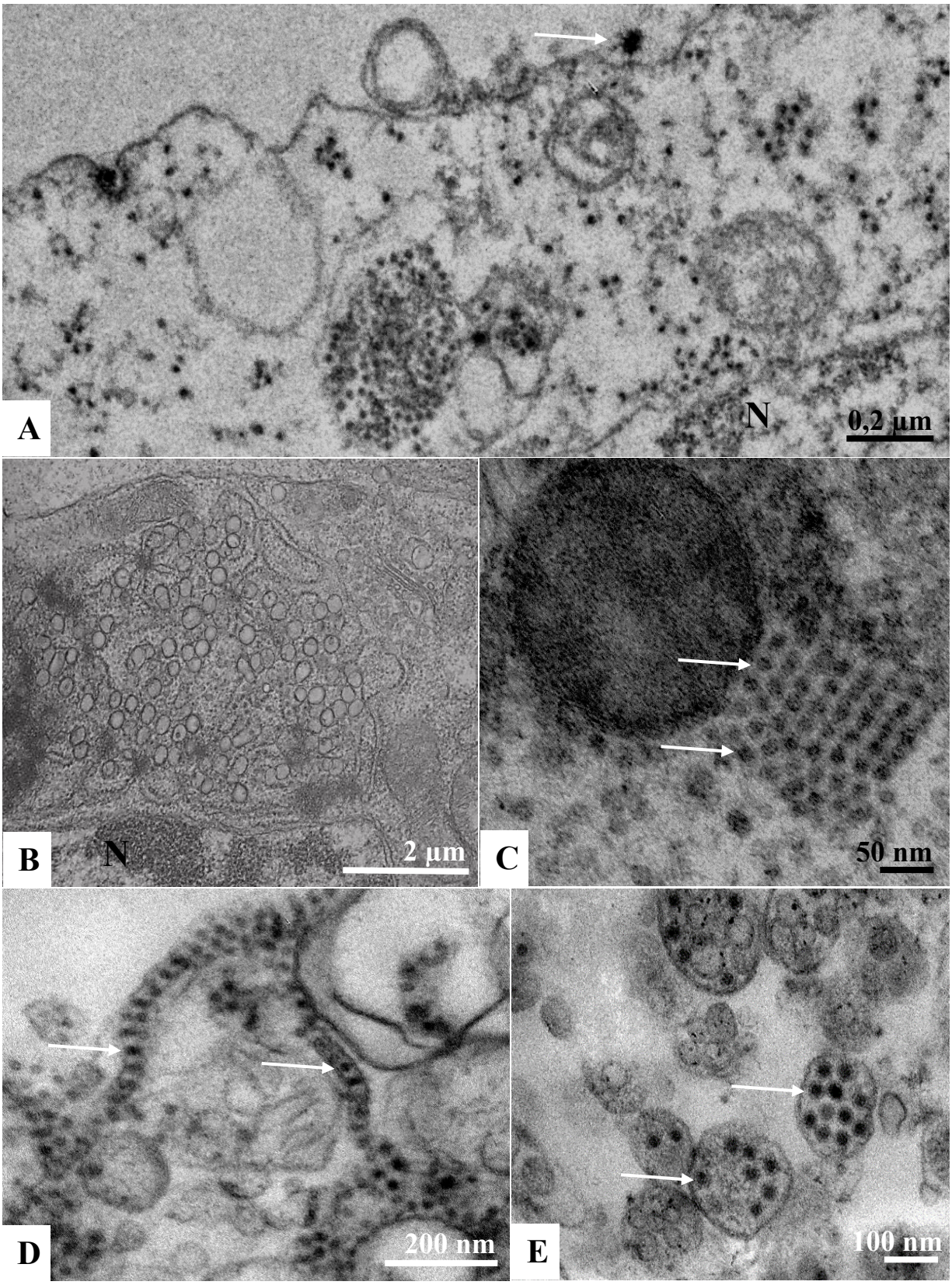



**Figure 3. YFV**. C6/36 cells, seven days post-infection, transmission electron microscopy. **A**: One yellow fever virus particle interacting with cellular receptors (arrow) and another one being internalized through an endocytic vesicle coated with clathrin (arrow head), **B**: Cell presenting tubular structures (asterisk) associated with the rough endoplasmic reticulum with YF particles-like inside (arrow) with diameter of approximately 200 nm, **C**: Yellow Fever nucleocapsids, **D**: Yellow Fever virus particles inside cisterns of the rough endoplasmic reticulum (arrow), **E**: Yellow Fever virus inside cytoplasmic vesicles (arrow). Nucleus (N).

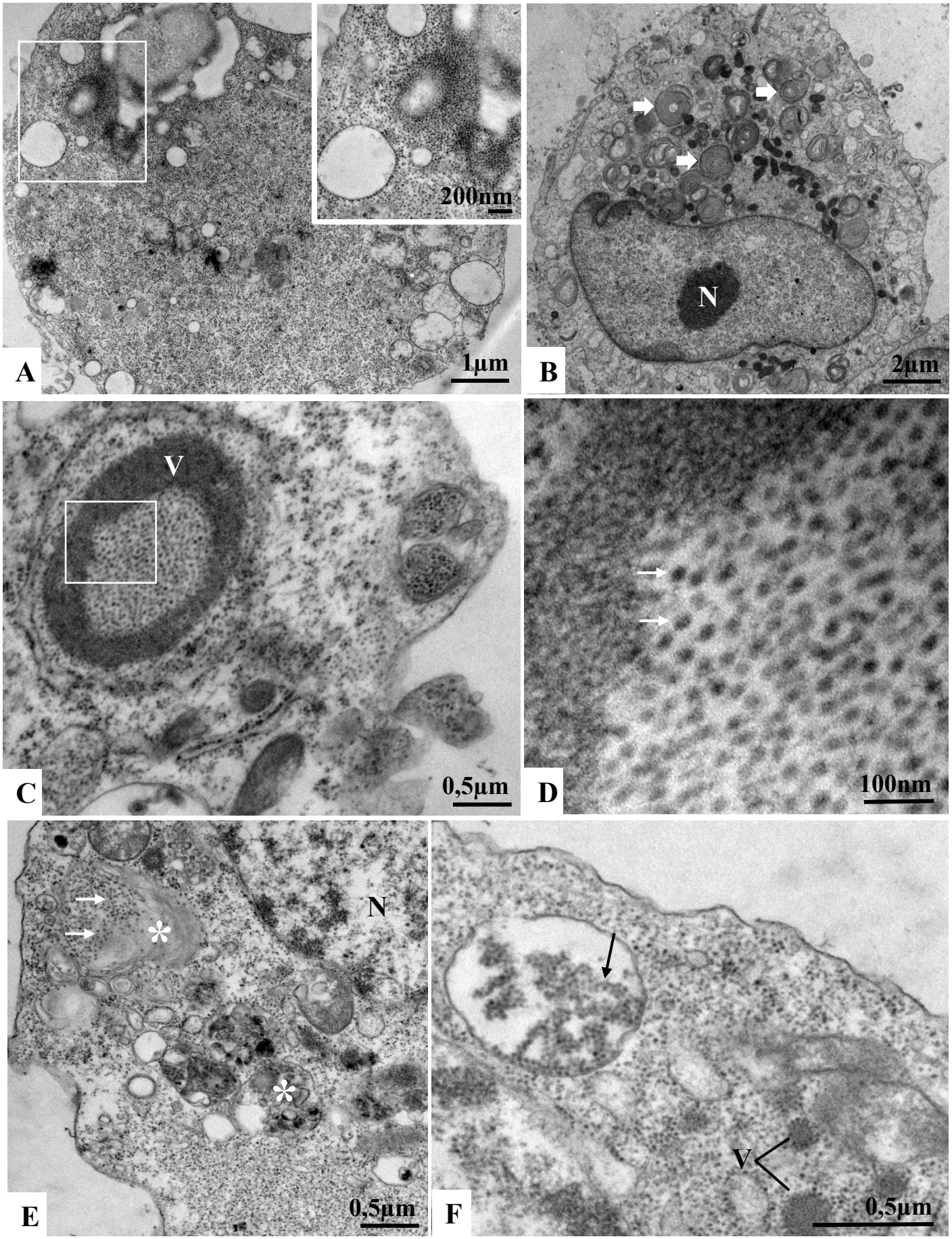



**Figure 4. ZIKV.** Infected C6/36 and Vero cells, transmission electron microscopy. **A**: Thickened ribosomes

(marked area) [C6/36 cells, 24hr post-infection], **B**: Numerous myelin figures and phagosomes (arrow) [Vero cells, 72 hours post-infection], **C**: Viroplasm-like perinuclear compartment (V) containing ZIKV particles in their lumen (arrow) [C6/36 cells, 48hr post-infection], **D**:Inset of marked area of the figure C, **E**: ZIKV particles (arrow) in lysosomes (asteristic) [C6/36 cells, 48 hours post-infection], **F**: ZIKV particles inside a RER vesicle (arrow) [C6/36 cells, 48hr post-infection]. Nucleus (N), mitochondria (M).

**Alphavirus:** Chikungunya

**A. Virus particles morphology** (Figure 5)

Ultrastructural analysis showed virus particles and its replication in C6/36 and Vero cells when inoculated with CHIKV. The CHIKV particles have a spherical shape with an approximate diameter of 50-60 nm and an evident envelope structure (figures 5A, 5B).

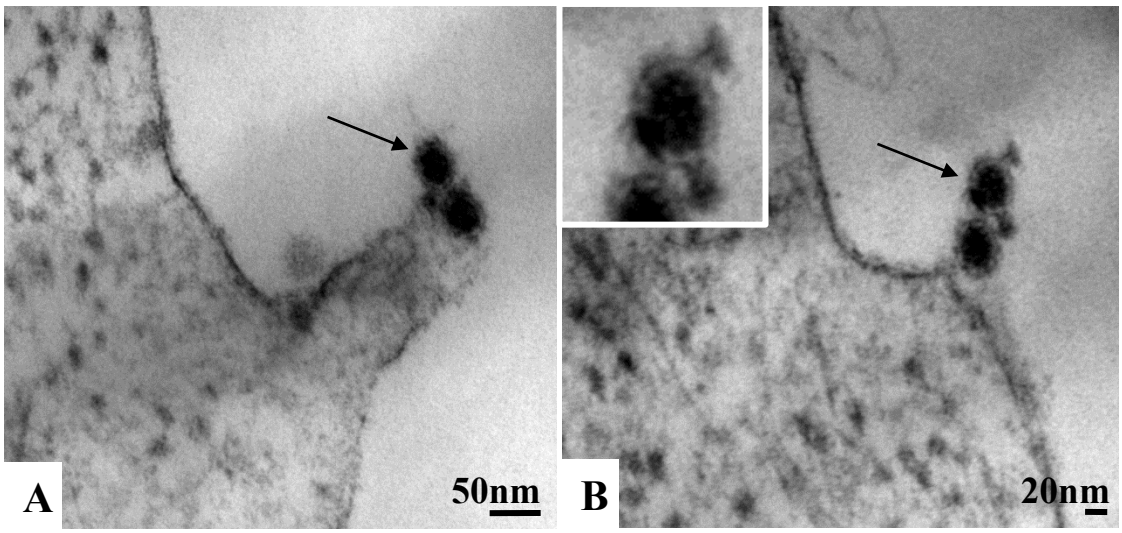
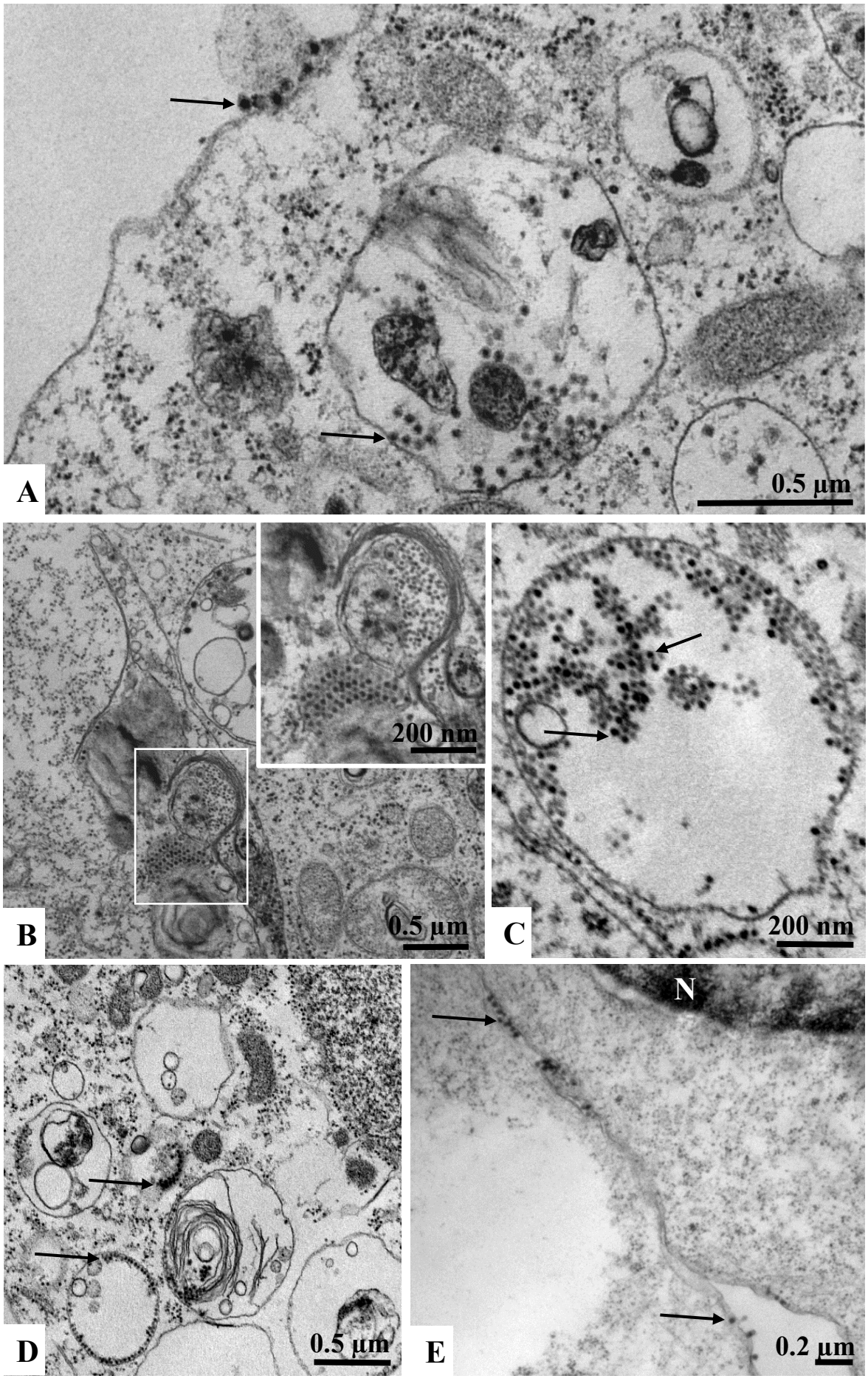



**Figure 5.** Chikungunya virus particles (arrow), Vero cells 72hr post-infection, transmission electron microscopy. A: Two viral particles budding from the cell membrane (arrow), **B**: Virus particles attached to the cell membrane (arrow); inset: higher magnification showing virus particles and its envelopes (arrow heads).

**B. Virus Morphogenesis** (Figure 6)

C6/36 and Vero cells infected with CHIKV showed that the virus particles was internalized mainly by clathrin-mediated endocytosis (Figure 6A). Viral nucleocapsids were detected inside and outside vesicles (Figure 6B). Virus particles occur inside unit-membrane coated cell compartments (Figures 6C e 6D). Release of CHKV particles by budding from the cell membrane was observed (Figure 6E).


A
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B
200 nm
0.5 µm
C
200 nm
D
0.5 µm
N
E
0.2 µm

**Figure 6. CHKV.** C6/36 and Vero cells, 72hr post-infection, transmission electron microscopy. **A:** Particles of Chikungunya virus attached to the cell membrane (arrow) and inside a cell vesicle (asterisk) [C6/36 cell], **B:** Nucleocapsids in cytoplasm (marked area), [C6/36 cells], **C/D:** Virus particles (arrows) associated with cell membranes [C6/36 cells], **E:** Viral particles budding from the cell membranes (arrows) [Vero cells]. Nucleus (N).

## DISCUSSION

The simultaneous circulation of different arboviruses (DENV, ZIKV, YF and CHIKV) in Brazil is of extreme concern to public health since the effect of the cocirculation and coinfection of these viruses on severity of the disease needs to be further investigated. Brazil is endemic for many arboviruses, and recently several cases have been reported. As an example, in 2017 Brazilian Health Ministry registered an incidence mean rate of 151.3 cases per 100,000 inhabitants for DENV, 249.6 for CHIKV and 9.3 for ZIKV in the Northeastern region. In the same period, the state of Pernambuco reported incidence rates of 95.5 cases per 100,000 inhabitants for DENV, 20.8 for CHIKV and 0.4 for ZIKV (Ministério da Saúde, Brasil, 2018). Morphological ultrastructural aspects and morphogenesis of these arboviruses currently circulating in Brazil were here analyzed using transmission electron microscopy.

DENV infection cycle initiates with the virus attachment to the target cell through the interaction between viral surface proteins and attachment/receptors molecules on the cell surface. This interaction allows the internalization of the virus particle, generally involving receptor-mediated endocytosis (Mukhopadhyay et al., 2005; Cruz-Oliveira et al., 2015). The present study demonstrated that DENV, YFV and ZIKV attachment is similar to other flaviviruses (Figures 3A and 6A) and the internalization of the viral particle was observed early inside endocytic vesicles coated with clathrin (Figure 3A). The DENV-1, -2, -3 -4, YFV and ZIKV particles presented morphology and diameter similar as observed in previous studies (Kostyuchenko et al., 2013; Prasad et al., 2017; Yu et al., 2008; Zang et al., 2003). No morphological differences were observed between the DENV serotypes.

In the present study, C6/36 and Vero cells infected with DENV-1, -2, -3 and -4 (Figure 2A) and ZIKV presented spherical and tubular structures associated to the rough endoplasmic reticulum and viral particles ontogenesis inside the rough endoplasmic reticulum cisterns, as illustrated for DENV-2 in Barth (2000). This is in agreement with

other reports about flaviviruses such as DENV, West Nile virus, Japanese Encephalitis virus, Tick-borne encephalitis virus and ZIKV (Barth, 1992; Mackenzie et al., 1996; Ng et al., 1994; Barreto-Vieira et al., 2017; Cortese et al., 2017; Morita and Masashi, 2019; Gillespie et al., 2010; Junjhon et al., 2014; Miorin et al., 2013; Welsch et al., 2009). These tubular structures with an inner vesicle diameter of approximately 200 nm were observed here in C6/36 cell cultures infected with YFV (Figure 3E). Since DENV tubular structures were shown to be associated with dsRNA (Junjhon et al., 2014; Welsch et al., 2009), these structures were believed to be the site of RNA replication. Single-stranded positive-sense RNA viruses are known to have properties that deform the endoplasmic reticulum membrane and create organelle-like compartments called viral replication-organelles (Romero-Brey and Bartenschlager, 2014; 2016). Studies using electron microscopy found endoplasmic reticulum-derived DENV-induced vesicles, convoluted membranes, and virus particles, and detected double-stranded RNA, a presumed marker of RNA replication, beside the virus-induced vesicles and tubules. Electron tomography showed DENV-induced membrane structures to be part of an endoplasmic reticulum-derived network (Welsch et al., 2009). Knowledge of cellular and viral factors participating in distinct steps of flaviviruses replication cycle is significant to the development of preventive and therapeutic strategies for dengue combat.

The flavivirus viral genome is of a single-strand positive sense RNA that functions as a messenger RNA, being subsequently translated by the cell machinery thus generating the viral proteins. Afterwards, the genome is replicated and new RNA copies are incorporated into nascent particles. The assembly of DENV occurs inside the rough endoplasmic reticulum and virions were then transported through the trans-Golgi network and secreted (Mukhopadhyay et al., 2005; Cruz-Oliveira et al., 2015). These studies were corroborated in the present, since the DENV-1, -2, -3, -4 and YFV presented enveloped nucleocapsids inside the cisterns of the rough endoplasmic reticulum (Figures 2B, 2C, 3D) and in cytoplasmic vesicles (Figures 2D, 3E).

C6/36 and Vero cells infected with ZIKV showed large viroplasm-like compartments (Figures 4C), localized in the perinuclear area next to the peripheric rough endoplasmic reticulum and microtubules. At the same time, large amounts of ribosomes, and mitochondria were recruited. ZIKV particles inside lysosomes (Figure 4D) and the rough endoplasmic reticulum (Figure 4F), as well as viral nucleocapsids inside the viroplasm-like structures (Figures 4C) were observed. These findings are similar to

those observed by Barreto-Vieira et al. (2017) demonstrating that susceptibility of C6/36 and Vero cells to ZIKV infection and that part of the replicative cycle might occur within viroplasm-like structures. This has not been previously seen in other flaviviruses infected cells. Subsequent studies demonstrated that ZIKV infection in both human hepatoma and neuronal progenitor cells induced drastic structural modifications of the cellular architecture (Cortese et al., 2017). ZIKV infection causes a drastic reorganization of microtubules and intermediate filaments forming cage-like structures surrounding the viral replication factories. Viroplasms are constituted during virus morphogenesis, when components such as replicase enzymes, virus genetic material, and host proteins were required for replication, and thereby increase the efficiency of replication (Christopher et al., 2007*).* The viral replication, protein synthesis and assembly require a considerable amount of energy, provided by large clusters of mitochondria at the periphery of viroplasms. The virus factory is often enclosed by a membrane derived from the rough endoplasmic reticulum or by cytoskeletal elements (Moshe and Gorovits, 2012). Viroplasms have been observed in cauliflower mosaic virus, rotavirus, vaccinia virus, and the rice dwarf virus (Eichwald et al., 2012; Majerowics et al., 1994; Szajner et al., 2001) and mimivirus and reovirus (Marie et al., 2007). Futher studies should be undertaken to understand the role of viroplasm-like compartment in the ZIKV replication cycle.

C6/36 and Vero cells cultures when inoculated with CHIKV, the virus particles were internalized mainly by clathrin-mediated endocytosis (Figure 6A) as reported in other studies also (Sourisseau et al., 2007; van Duijl-Richter et al., 2015; Bernard et al., 2010). Viral particles replication seems to occur inside unit-membrane coated cell compartments (Figures 6C e 6D). Studies demonstrated that negative-strand synthesis was linked to formation of viral replication units termed spherules, which are small, vesicular structures that form at the plasma membrane and serve as the site of genomic viral RNA replication. The nonstructural proteins are thought to localize at the neck of the spherules, which house dsRNA intermediates, protecting them from degradation and recognition by cellular pattern-recognition receptors (Wu et al., 2013; Frolova et al., 2010; Spuul et al., 2010; Kujala et al., 2001; Thaa et al., 2015; Utt et al., 2016). As infection proceeds, spherules are internalized to form large cytopathic vacuoles, which contain markers from endosomal and lysosomal membranes (Silva and Dermody, 2017; Spuul et al., 2010; Kujala et al., 2001). The release of virus particles is by budding from the cell membrane (Jin et al., 2018), observed also in our studies (Figure 6E).

It was here demonstrated that the morphology and morphogenesis of the currently circulating arboviruses (DENV, ZIKV, YF and CHIKV) in Brazil are similar to the data resulting from previous studies. However, we understand that there needs to be more studies to determine mainly the role of viroplasma-like structures in ZIKV replication.

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