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Dengue and Zika flaviviruses in bats
Dengue y Zika flavivirus en murciélagos

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Bats are natural reservoir hosts of a diverse viruses that affect humans. However, the role of bats as reservoirs for dengue (DENV) and Zika (ZIKV) flaviviruses is still controversial. Molecular and serological detection in several species suggests receptivity. Backwards, other works suggest that chiropters are not receptive as no antibodies or viruses were detected. This review examines the controversy, analyzing laboratory and field studies and 4 publications about the experimental infection of *Artibeus* sp. bats with DENV and ZIKV. Contradictory reports about the susceptibility of chiropter cell lines to replicate DENV may be due to the phylogenetically divergent origin of cells used, as in a study reporting viral production, proceeds from Old World bats (Megachiroptera) and in studies reporting not viral production, proceeds from neotropical bats (Yangochiroptera). Serological contradictory reports may be due to the used tests. The most accurate test is the Sero-Neutralization Test (SNT). Reports using Hemagglutination Inhibition (HIT) or Enzyme-Linked Immunosorbent Assay (ELISA) could give false positives. Thereby, all 4 experimental infections analyzed, used neotropical (Yangochiroptera) *Artibeus* sp. bats, and all reported defective or no replication of both flaviviruses by RT-PCR reference test. Considering SNT as a gold standard, no seroconversion was found. However, histopathological alterations were reported in organs of experimentally infected bats with both flaviviruses. These results suggest that bats of the genus *Artibeus* sp. are not efficient amplifiers or reservoirs of DENV or ZIKV. Nevertheless, both human flavivirus could cause alterations. Other bat species need to be studied.

Key words: *Artibeus* bats; flaviviruses; RT-PCR reference test; sero-neutralization test; Zika and dengue.

Los murciélagos son reservorios naturales de virus que afectan a los humanos; empero, su papel como reservorios del dengue (DENV) y Zika (ZIKV) es controversial. Su detección molecular y serológica en varias especies sugiere receptividad. Al contrario, otros estudios sugieren que no son receptivos, pues no detectan anticuerpos ni virus. Se examina dicha controversia, analizando estudios de laboratorio y de campo y 4 publicaciones sobre infección experimental de murciélagos *Artibeus* sp. con DENV y ZIKV. Informes contradictorios sobre la susceptibilidad de las líneas celulares de quirópteros para replicar el DENV pueden deberse al origen filogenéticamente divergente de células utilizadas; en un estudio que reporta producción viral, proceden de murciélagos del Viejo Mundo (Megachiroptera). Estudios en los que no se detecta producción, proceden de murciélagos neotropicales (Yangochiroptera). Los informes serológicos contradictorios pueden deberse a las pruebas utilizadas. La prueba más precisa es la seroneutralización (SNT). Estudios en los que se emplearon la inhibición de la hemaglutinación (HIT) o ensayos de inmunoabsorción ligado a enzimas (ELISA) podrían dar falsos positivos. Así, las 4 infecciones experimentales analizadas en este trabajo utilizaron murciélagos neotropicales (Yangochiroptera) *Artibeus* sp. y en las 4 se reportó replicación viral ineficiente para ambos flavivirus mediante RT-PCR. Considerando la SNT como un estándar, no se detecta seroconversión; empero, se reportaron alteraciones histopatológicas en murciélagos infectados experimentalmente. Los resultados sugieren que murciélagos del género *Aribeus* sp., no son amplificadores eficientes del DENV o ZIKA; empero, ambos flavivirus podrían causarles alteraciones. Faltaría estudiar otras especies.

Palabras clave: Flavivirus; Murciélagos *Artibeus*; Prueba de referencia RT-PCR; Prueba de virus-seroneutralización; Zika y dengue.

Dengue virus (DENV) and Zika virus (ZIKV) belong to the genus *Flavivirus* inside the *Flaviviridae* family (ICTV 2024). These viruses affect around 390 millions of people worldwide and induce 2 of the most important human vector-borne diseases (Bhatt *et al.* 2013). Dengue is a disease that has been known for a long time. The first reports of major epidemics of an illness thought to possibly be dengue occurred in Asia, Africa, and North America in 1779 and 1780 (Gubler 1998). Nowadays DENV has affected a large number of countries in Asia, Africa, and The Americas,

mainly in tropical and subtropical areas. On the other hand, ZIKV was isolated for the first time in 1947 from a rhesus monkey and the infection in humans was first described in Nigeria, Africa in 1954 (Musso and Gubler 2016). In the second half of the 20th century, only around 20 human cases have been reported. ZIKV was first reported in the Americas in 2015 and since then it spread throughout the continent causing more than 850,000 human cases (Hennessey *et al.* 2016; Zhang *et al.* 2017). Both flaviviruses can establish sylvatic cycles in which mammals and wild vectors are involved, thus becoming reservoirs of the disease (Figueiredo 2019).

Chiropters are significant natural hosts of various viruses that can be transmitted to humans as rabies and other Lyssaviruses, Hendra, and Nipah viruses, and have been also identified as the likely reservoir for severe acute respiratory syndrome (SARS) coronavirus (Li *et al.* 2005) and Marburg virus. Furthermore, several studies have indicated that bats have unique defense mechanisms that allow them to be persistently infected with viruses without major pathological consequences (Brook and Dobson 2015; Subudhi *et al.* 2019).

It has been suggested that bats may play a role as alternatives hosts in the transmission of flaviviruses *(i.e.*, Japanese encephalitis and St. Louis encephalitis viruses; Mackenzie *et al.* 2016). However, the exact involvement of bats in the epidemiology of DENV and ZIKV is still a matter of controversy. Molecular and serological detection of flaviviruses in bats in several reports suggest exposure to DENV and ZIKV in the wild (Aguilar-Setién *et al.* 2008; De Thoisy *et al.* 2009; Machain-Williams *et al.* 2013; Sotomayor-Bonilla *et al.* 2014; Calderón *et al.* 2019; Calderón *et al.* 2021). On the contrary, other works suggest that chiropters are not receptive to these flaviviruses (Cabrera-Romo *et al.* 2014, 2016; Moreira-Soto *et al.* 2017; Bittar *et al.* 2018). This review analyzes the controversy, considering some representative works published on the subject and others carried out by our group, as well as 4 experimental infections performed in *Artibeus* sp. bats.

Findings indicating susceptibility of bats to DENV and ZIKV. Based on previous reports about bats' susceptibility to DENV in different areas of the Americas (Reagan and Brueckner 1952; Platt *et al.* 2000), our group conducted a study of neotropical bats from DENV-endemic areas of the Pacific and Gulf coasts of México during the years 2006 to 2008 (Aguilar-Setién *et al.* 2008). In this study, 162 samples were analyzed proceeding from 5 families, 12 genera, and 19 different species of bats it was founded that 19 (12 %) showed DENV antibodies. Besides, sequences of DENV serotype 2 were also detected by RT–PCR in 4 samples from 3 bat species: the frugivorous *Artibeus jamaicensis* (2/9) and *Carollia brevicauda* (1/2), and the insectivorous *Myotis nigricans* (1/11). Supporting this finding, De Thoisy *et al.* (2009) reported DENV RNA in 14 species of neotropical bats sampled between 2001 and 2007 in French Guyana. Later Machain-Williams *et al.* (2013), Sotomayor-Bonilla *et al.* (2014) and Calderón *et al.* (2019) reported antibodies and RNA of DENV in bats in México and Colombia. Besides, an *in vitro* study showed that Old-World fruit bat cells (*Pteropus alecto*, *Eonycteris spelaea* and *Cynopterus brachyotis*) can be infected with DENV, producing high titers of virus with limited immune cellular responses but a minimal interferon (IFN) response in these bat cells (Irving *et al.* 2020). These findings suggest the possibility that bats could be transient hosts in the epidemiological cycle of DENV.

For ZIKV, experimental infections and field studies performed in the middle of the 20th century documented the probable susceptibility and disease development in African and American bat species (*Myotis lucifugus*, *Tadarida* spp. and other non-specified species; Reagan *et al.* 1955; Shepherd and Williams 1964; Simpson *et al.* 1968). More recently Torres-Castro *et al.* (2021) reported evidence of West Nile Virus (WNV) and ZIKV natural infection in bats from Yucatán, México. Besides, an experimental infection in a breeding colony of *A. jamaicensis* bats described the development of histological lesions, the detection of RNA of the virus in a few samples, and seroconversion in some studied animals (Malmlov *et al.* 2019). These results led to suggest the possibility that these animals may have a role in ZIKV epidemiology and that may even endanger bat populations. The reports of bat susceptibility to ZIKV from the mid-20th century, the experimental infection performed by Malmlov *et al*. in 2019, and the rapid spread of ZIKV in the American Continent after 2015 allow us to consider that ZIKV could adapt to new vertebrate wildlife hosts. As happened with the yellow fever virus (YFV; Goes De Jesus *et al.* 2020), ZIKV might establish a sylvatic cycle until there is a large enough naïve population of wild species.

Findings indicating that bats are not susceptible to DENV and ZIKV. No evidence of sustained replication of DENV was documented in experimentally inoculated *A. jamaicensis* bats with DENV serotypes 1 or 4 by Cabrera Romo *et al.* (2014). This same group of researchers in 2016 reported no evidence of DENV infections in several species of wild bats captured in central and southern México (Cabrera-Romo *et al.* 2016). Contrary to what was reported by Irving *et al.* (2020; a high replication of DENV in flying foxes), *in vitro* studies conducted by Moreira-Soto *et al.* (2017), infecting now neotropical bat cell lines (*A. jamaicensis* and *Desmodus rotundus*) with DENV, showed they are inadequate hosts as infected cells showed null replication of the virus (Moreira-Soto *et al.* 2017). Besides these same authors, in a peridomestic study, in Costa Rica, reported only limited exposure of bats, likely due to proximity to humans and consumption of DENV vectors, since although the virus was detected by RT-PCR in very few animals, it was not possible to isolate it in cell lines and in addition, no evidence was found that *Aedes* spp. mosquitoes analyzed in the area would feed on bats (Vicente-Santos *et al.* 2017).

Regarding ZIKV, Bittar *et al.* (2018), reported a lack of serological and molecular evidence of ZIKV infection in 103 bats from 4 families and 19 species in Brazil. Nevertheless, in the experimental infection in particular in *A. jamaicen-*

sis bats with ZIKV, carried out by Malmlov *et al.* (2019), they conclude that bats may have a role in ZIKV epidemiology, a very low percentage of ZIKV RNA detection was reported in the experimentally infected bats. Among a series of samples of organs, blood, and secretions taken from 9 *A. jamaicensis* bats experimentally inoculated with ZIKV, only in 3 samples the virus could be detected by RT-PCR (in a brain sample from a bat taken at 2 days post-inoculation (dpi), in a urine sample taken from another individual at 3 dpi, and in a final urine sample from a third individual taken at 5 dpi. The low percentage of detection of ZIKV RNA reported in the mentioned work, together with the high sensitivity of the RT-PCR test, permit doubt about the *in vivo* efficiency of *A. jamaicensis* bats to replicate ZIKV as sustained virus replication was not demonstrated as occurs in the true susceptible species as humans and primates.

Experimental infections with DENV and ZIKV *Flavivirus*. Since the controversies found in the literature, and to elucidate the *in vivo* susceptibility of genera *Artibeus* bats (abundant bat genera in México) to DENV and ZIKV, our group carried out experimental infections with each one of these Flaviviruses using different groups of bats. Concerning DENV, an experimental infection in 23 *Artibeus intermedius* bats (known also as *A. lituratus intermedius* bats) captured in the wild in Morelos State in México (18° 54´ 23´´N, 98° 58´ 13´´W), with different viral loads of DENV-2 was performed (Perea-Martínez *et al.* 2013). Unexpectedly, a high percentage (43 %) of bats developed macroscopic lesions consisting of bruises (hemorrhage) on the chest and/or on the wings (Figure 1). Histological analyses showed structural alterations in the spleen and bleeding in the liver and intestine, but the virus was not detected by semi-nested RT-PCR in any of the histological altered or not tissues, except for one infected bat kidney. In sera, the viral RNA was detected by semi-nested RT-PCR in 39 % of bats, but only 8 % of bats seroconverted. Overall, these data indicate that DENV-2 has poor and non-continuous virus replication in these animals as the RNA detected in sera could be residual RNA of the inoculum.

Concerning ZIKV an experimental infection was conducted by our group using 12 *A. lituratus* bats, captured also in Oaxtepec, Morelos, México, in the wild (Aguilar-Setién *et al.* 2023). The results were consistent with the previous work performed by Malmlov *et al.* (2019). We found histopathological alterations mainly in the testicles, ovaries, and central nervous system (CNS) of some infected animals, but as described for DENV in the study performed by Perea-Martínez *et al.* (2013), ZIKV RNA was not detected by RT-PCR in any altered or not tissues of the infected animals. ZIKV was detected by RT-PCR only in 2 urine samples proceeding from experimentally infected animals. Additionally, as it was reported also for DENV, we observed that some infected animals showed macroscopic lesions such as hemorrhages on the chests, wings, and bladder. In Malmlov *et al.* (2019) work, antigens of ZIKV were detected by an immunohistochemical test on histopathological altered tissues (testes, salivary glands, lung) of experimentally infected animals. Interestingly, in none of these organs in which the antigen was located, ZIKV RNA could be amplified by RT-PCR. To verify these results, our group subsequently searched for antigens through the Immunofluorescence (IF) test in frozen tissues, recovered from bats experimentally infected previously (Aguilar-Setién *et al.* 2023). According to what was reported by Malmlov *et al.* (2019) we found antigens in some histopathological altered tissues but also it was not possible to detect viral RNA by RT-PCR.

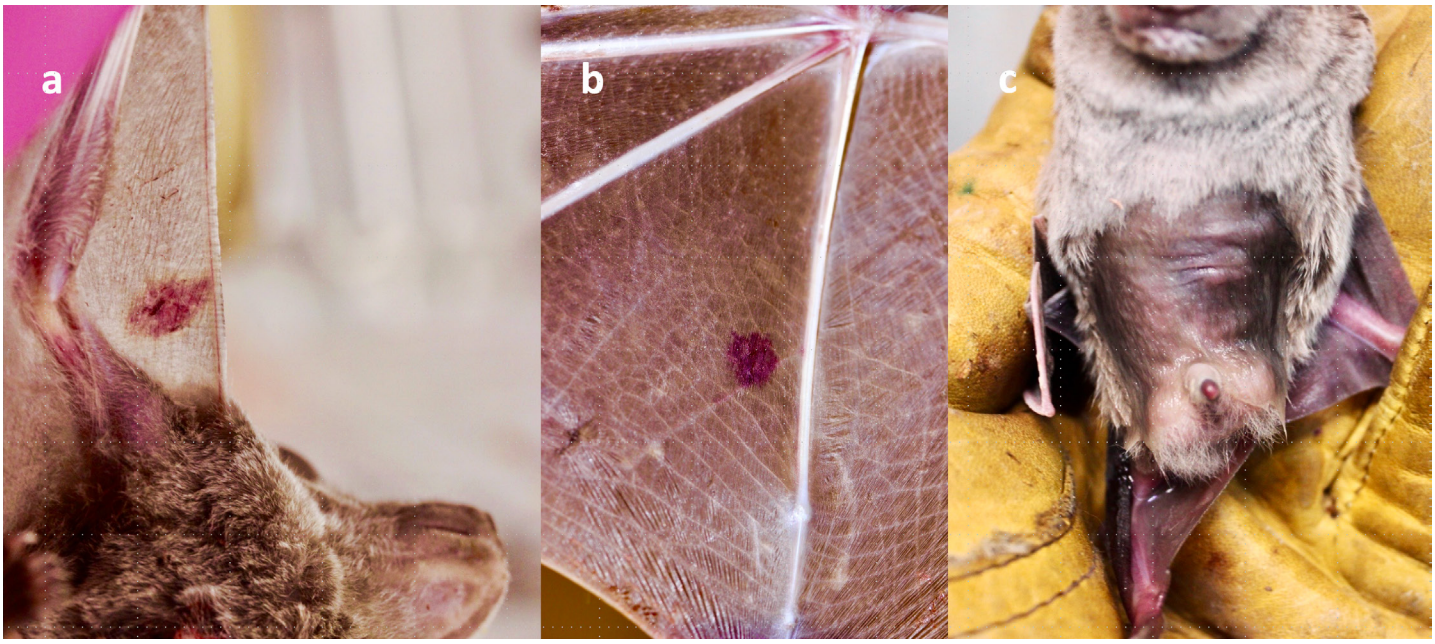

Figure 1. Bats inoculated with DENV-2 developed hematomas. a) and b) Wings of bats showing bruises 3 days post-DENV inoculation; c) ventral region of a bat showing bruises, 3 days post-DENV inoculation. Photographs: A. Aguilar-Setien. Images available at balantiopterix@gmail.com.

Microscopic lesions caused by DENV and ZIKV inoculation. In Malmlov *et al.* (2019) using ZIKV microscopic lesions reported were: infiltrates and microscopic hemorrhage in the lungs, cellular infiltrates and cardiomyocyte necrosis in the heart, degeneration and lymphocyte infiltration in testes, neuronal degeneration in brain and salivary gland inflammation. In Aguilar-Setién *et al.* (2023) using ZIKV, microscopic lesions reported were: lymphocyte infiltration in testes, ovaries, and uterus; mature lymphocyte decrease, and immature lymphocyte increase in spleen; hemorrhage and leukocyte infiltration in bladder, stomach and wing skin. Also, in Perea Martínez *et al.* (2013) study using DENV granuloma-like alterations in the spleen were reported. In Cabrera-Romo *et al.* (2014) no microscopic lesions were reported because they were not searched (Table 1).

The order Chiroptera has an enormous diversity of species, only surpassed in number by rodents within mammals, and that the same susceptibility to an infectious agent cannot be generalized for all species. The studies carried out to elucidate the susceptibility of bats to 2 Flaviviruses (DENV and ZIKV) that significantly affect humans, resulted in scarce and fragmented if we consider the great diversity of bat species existing in the world.

In the work performed by Moreira-Soto *et al.* (2017), cell lines used to DENV isolation proceed from neotropical bats: 2 from the Phyllostomidae family (*A. jamaicensis* and *D. rotundus*) and one from the Molossidae family (*Molossus sinaloae*), both families, belonging from the Yangochiroptera suborder. Regarding Irving's work (Irving *et al.* 2020), cell lines used, proceed from *P. alecto*, *E. spelaea* and *C. brachyotis*, 3 species of the Old World bats, belonging to the Pteropodidae family of the Megachiroptera suborder. It is evident that the source of cell lines used in each one of the two works are phylogenetically divergent (Agnarsson *et al.* 2011) and the contradictory results obtained may be due to phylogenetic differences.

The sensitivity and specificity of the tests used in each of the works consulted may be a factor in the reported divergences. In virology, it is accepted that one of the most specific tests that indicates protection and contact against a specific virus is the Sero-Neutralization Test (SNT). SNTs are capable of providing high specificity among flaviviruses and are considered the gold standard in quantifying and detecting the levels of neutralizing antibodies (Chan *et al.* 2022). The Hemagglutination Inhibition Test (HIT) has the advantage of being easy to perform in places with few resources and was the first test used many years ago to detect antibodies against DENV. Nevertheless, it has been reported that the HIT test is unable to differentiate among the flaviviruses DENV, Japanese Encephalitis Virus JEV, and West Nile Virus WNV, therefore is less specific than the SNT (Nisalak 2015). In ELISA tests, sensitivity and specificity will depend on the quality of the antigens used in the plates. It is a test to be used when a large number of samples must be processed. To perform ELISA tests it is necessary to have an equipped laboratory that allows us to establish parameters and make accurate measurements. It is important to note that errors occur when there is insufficient blocking of the surface of a microtiter plate immobilized with antigen and this can lead to high false-negative or false-positive results (Guzmán *et al.* 2010; Sakamoto *et al.* 2018).

For ZIKV, DENV, and other arboviruses experimental infections and field studies in bats performed in the middle of the XX Century, were made using HIT for antibody detection (Reagan *et al.* 1955; Shepherd and Williams 1964; Simpson *et al.* 1968), hence, the positive results may not be as specific as expected. In 3 out of the 4 bat experimental infections studies analyzed in the present review, antibodies were measured by ELISA. In 2 out of the 3 studies using ELISA some positive results were reported (Table 1). While in the only one in which the SNT was used, no positive results were reported (Table 1). The positive results reported in the works using ELISA tests may be false positives, for the mentioned characteristics inherent to this test. Given the high specificity of the SNT, the negative results obtained in the work using this test would be more accurate.

Concerning virus detection in the 4 experimental infections works analyzed in this review, all used RT-PCR and in all, a low number of positive samples were reported without being able to conclude a sustained replication of the viruses used, in any case (Table 1). In the 2 experimental infections of bats with ZIKV over more than 60 samples (tissues, sera, plasma, and urine) taken throughout the infection, only in 4 urine samples and 1 tissue sample (brain), the virus could be detected by RT-PCR, confirming that there is no sustained virus replication. It must be considered that RT-PCR is a highly sensitive and specific method for virus genome detection and that the detection of some positive samples could be due to residual RNA of the original inoculum. It is noteworthy that, despite not demonstrating sustained viral replication, some animals presented microscopic lesions in some studied cases (Table 1). In addition to the fact that in any case viral RNA could be detected in any of the altered tissues of the experimentally infected bats, in Malmlov *et al.* (2019) the virus could not be isolated in cell cultures inoculated with sera samples of experimentally infected animals.

Finding some clinical alterations with scarce or no viral RNA detection could be somewhat contradictory, and one explanation could be the hypersensitivity phenomena produced by certain viral proteins and/or a reaction to the inoculum coadjuvant/diluent. Modhiran *et al.* (2015) reported an analogy between the cellular biology of bacterial lipopolysaccharides (LPS) and that of DENV nonstructural protein 1 (NS1). LPS and DENV NS1 interacts with Toll-like receptor 4 (TLR 4) on the surface of monocytes, macrophages, and endothelial cells, inducing the release of a range of cytokines and chemokines. These same cytokines circulate in the blood of patients with hemorrhagic Dengue syndrome, producing hemorrhages and tissue destruction (Halstead 2021). However, we do not

Table 1. Results obtained in 4 studies of experimental infection of bats with dengue (DENV) or Zika (ZIKV) flaviviruses. IC: Intracerebral route; IP: Intraperitoneal route; SC: Subcutaneous route; ND: Not Determined.

Bats inoculated	Inoculum	Route of inoculation and amount of inoculated virus	Virus detection	% Virus positive samples	Antibodies detection	Antibody positive samples	Macroscopic lesions	Microscopic lesions
Perea-Martínez *et al*. (2013)								
Wild-caught *Artibeus intermedius* *n* =12	DENV2 strain New Guinea-C	IC10^6 PFUs; IP 64, 10^6 and 1.7 X 10^7 PFUs	RT-PCR Lanciotti	Sera 39 % Tissues 1.7 %	Dengue IgG ELISA, Human GmbH Wiesbaden, Germany, using as 2d antibody Protein A peroxidase conjugate	22 %	Bruising on the chest Hematomas on wing skin	Granuloma-like alterations in spleen
			ELISA Platelia TM Dengue NS1 AG (Bio-Rad)	0 %				
Cabrera-Romo *et al*. (2014)								
Wild-caught *Artibeus jamaicensis* *n* = 38	DENV4, strain H241	SC 1.7 X 10^4 PFUs; IP 1.7 X10^4 PFUs; Mosquito bite ND	RT-PCR Lanciotti	Plasma 33 % (Suspected: weakly positive)	Competitive ELISA using Panbio Diagnostics plates	0 %	No lesions detected	ND
	DENV1, strain Hawaii	IP 4.8 X10^3 PFUs; Mosquito bite ND						
			ELISA Platelia TM Dengue NS1 AG (Bio-Rad)	Plasma 25 %				
Malmlov *et al*. (2019)								
Bred in captivity *Artibeus jamaicensis* *n* = 9	ZIKV strain PRVABC59	SC 7.5 X 10^5 PFUs	RT-PCR	Over about 30 samples of tissues, sera and urine, 3 resulted positive: 2 urine samples and 1 brain sample	ELISA using infected an fixes cells as antigen	33 % At low titers	No lesions detected	Infiltrates and microscopic hemorrhage in lungs; cellular infiltrates and cardiomyocyte necrosis in heart; degeneration and lymphocyte infiltration in testes; neuronal degeneration in brain and salivary gland inflammation
Aguilar-Setién *et al*. (2023)								
Wild-caught *Artibeus lituratus* *n* = 12	ZIKV strain Mer. IPN01	IP 2 X 10^5	RT-PCR	Over about 32 samples of tissues, sera and urine, 2 resulted positive: 2 urine samples	SNT	0 %	Hematomas on wing skin and in the bladder of one animal	Degeneration and lymphocyte infiltration in testes, ovaries and uterus; mature lymphocyte decrease and immature lymphocyte increase in spleen; hemorrhage and leukocyte infiltration in bladder, stomach and wing skin

know if this could be extrapolated to ZIKV; therefore, more research in this regard is necessary to better understand the immune system of bats against viral diseases. It would be interesting to prove this hypothesis by the inoculation of inactivated ZIKV or its components in our animal model. It is also possible that the observed macroscopic lesions (hemorrhage) and microscopic alterations could be due to factors as other microbiological and/or physical agents as in 3 out of 4 experiments, animals used have been captured in the wild and except for the agents studied, no tests were carried out to detect other pathogens (Perea-Martínez *et al.* 2013; Cabrera-Romo *et al.* 2014; Aguilar-Setién *et al.* 2023).

In conclusion, the results analyzed together, suggest that the bats species considered in these studies are not efficient amplifiers or reservoirs of DENV and ZIKV and may not have an important role in the transmission dynamics of these 2 medicals important flaviviruses. This statement is also supported by the fieldwork reported in Aguilar-Setién et al (2023), in which no viral RNA of ZIKV was detected in 2,056 individual blood serum samples from free-living neotropical bats of 34 genera collected in Perú, French Guiana, and Costa Rica, before (368 samples)

and during (1,688 samples) ZIKV pandemics (2010-2019). Conversely, although it seems that these important flaviviruses that affect humans do not replicate continuously in the studied bats, there is the possibility that they could cause some health alterations since histopathological lesions were found in the experimentally infected animals in 3 out of the 4 studies analyzed, and in the only one in which histopathological lesions were not mentioned they were not searched. Although we can not rule out the possibility that they did not happen.

It must be kept in mind that RNA viruses, due to their reduce genome size, are the known biological entities with the highest rate of mutation and adaptation (Mattenberger *et al.* 2021). Human agents could adapt and spread in wild animals. There are many examples of the zoonotic process (influenza, rabies, MERS-COV; Bengis *et al.* 2004), and there is no reason to believe that the opposite process cannot occur now or in the future.

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