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Evidence of the presence of the Zika virus in Mexico since early 2015

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Abstract To assess the possible circulation of Zika virus (ZIKV) prior to the first documented case in Mexico, we reanalyzed the stored samples from the states of Veracruz and Yucatán, which were originally collected to test for dengue (DENV) and chikungunya (CHIKV) but were negative for these viruses despite the symptomatology. The samples were originally collected between the 30 and 46 epidemiological weeks (EW) when the ZIKV was not yet declared as a Public Health Emergency of International Concern (PHEIC). From the total 4016 negative samples, a total of one hundred samples, 50 from Veracruz ($CHIK^-$ $DENV^-$) and 50 from Yucatán (4 $CHIK^-$ $DENV^-$ and 46 $CHIK^-$ or $DENV^-$), were tested for Zika virus by using RT-PCR. Results showed that in Veracruz and Yucatán, 20 % (10/50) and 70 % (35/50) were, respectively, ZIKV positive, indicating unequivocally the presence of ZIKV at least since July 2015. We also tested non-confirmed suspect measles cases from early 2015 for ZIKV by RT-PCR. Remarkably in 11 Mexican states, 86 % (18/21) were positive with the earlier symptoms onset as early as May 2015. Finally, RT-PCR analyses on RNA extracted from *Aedes aegypti* mosquitoes captured from January to March 2015 showed the presence of ZIKV, strongly suggesting that the vector was already carrying the virus at the start of 2015.

Keywords Zika virus · Mexico · RT-PCR · Retrospective analysis

The emergence of Zika virus disease (ZIKV) in the Americas has been widely documented [1, 2]. The first imported Zika virus disease case in Mexico was identified in October 2015 [Epidemiological week (EW) 41]. By December 2015, 1 imported and 15 autochthonous cases of Zika virus disease were confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR) [3]. The Mexican Public Health Laboratory Network (RNLSP) has reported an accumulated number of 1285 cases of Zika Virus infections at the 29th EW 2016 in at least 15 states of Mexico [4]. To evaluate the possible circulation of Zika virus prior to the first documented case, we reanalyzed the stored samples from Veracruz and Yucatán, which were originally collected to test for dengue (DENV) and chikungunya (CHIKV) by RT-PCR; we tested specimens that were negative for these viruses despite the symptomatology (in this way, we excluded the coinfections of CHIKV with ZIKV or DENV with ZIKV) and samples that were negative for CHIKV or DENV. The samples were collected between the 30 and 46 epidemiological weeks when the ZIKV was not yet declared a PHEIC; therefore, those samples were then not tested for ZIKV. From the total 4016 negative samples, a total of one hundred samples, 50 from Veracruz ($CHIK^-$ $DENV^-$) and 50 from Yucatán (4 $CHIK^-$ $DENV^-$ and 46 $CHIK^-$ or $DENV^-$), were then tested for Zika virus by real-time RT-PCR, using the Superscript III system (Invitrogen, Carlsbad, CA, USA)

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and primers and probes previously reported [5]. Using Zika virus nucleotide sequence data in the Primer3Plus web interface [5], we amplified a 760-bp fragment with the following primers for partial characterization of viral NS5 coding gene: ZikV9113Fwd TTYGAAGCCCTTGGATT CTT and ZikV9872Rev CYCGGCCAATCAGTTCATC. We used the QIAGEN One-Step RT-PCR Kit as follows: reverse transcription at 50 °C for 30 min, followed by an activation step at 95 °C for 15 min and 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. We sequenced several amplicons in the ABI PRISM 3130xl Genetic Analyzer instrument using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). To validate the test results, a positive control (RNA synthesized from a reference strain provided by InDRE) and a negative control (without template) were included in each RT-PCR run. It is important to note that all samples were collected from patients with less than 5 days after the symptoms onset. Results showed that in Veracruz and Yucatán, 20 % (10/50) and 70 % (35/50) of the samples were, respectively, ZIKV positive, indicating unequivocally the presence of ZIKV at least since July 2015 in the Gulf of Mexico and the Yucatán Peninsula (Fig. 1a).

Given the overlapping symptomatology of Zika virus disease and measles [6], we also tested non-confirmed suspect measles cases from early 2015 (stored at −20 C) for Zika virus by RT-PCR. Remarkably in 11 Mexican states, 86 % (18/21) were positive for ZIKV with the earlier symptoms onset as early as May 2015 (EW 19; Fig. 1a), with sixteen samples corresponding to 1- to 7-year-old children from ten Mexican states. These results indicate that syndromic classification of exanthematous diseases and Zika fever should be revised.

Moreover, as part of an active entomo-virological surveillance for dengue through the RNLSP, RT-PCR analyses of RNA extracted from *Aedes aegypti* mosquitoes (macerated groups of no more than 25 specimens) collected at the southern state of Guerrero were performed. The capture of mosquitoes was carried out since January 2015. Briefly, adult female mosquitoes were grouped in pools of 4 up to 25 (*mean* = 9.3) specimens. Each pool was homogenized with a tissue disruptor (Qiagen) and centrifuged at 2000 rpm for 5 min at 4 °C. Viral RNA was extracted from the supernatant using QIAamp Viral RNA minikit (QIAGEN, Inc., Valencia, CA) and stored at −20 °C until further use. Each pool was processed for the presence of ZIKV using the above-described RT-PCR protocol. To validate the test results, a positive control (RNA synthesized from a reference strain provided by InDRE) and a negative control (without template) were included in each RT-PCR run. The threshold (CT) was determined based on the positive and negative controls, with a detection limit of ≤39 cycles.

It is noteworthy that ZIKV positive results were found from the first tested sample, strongly suggesting that the

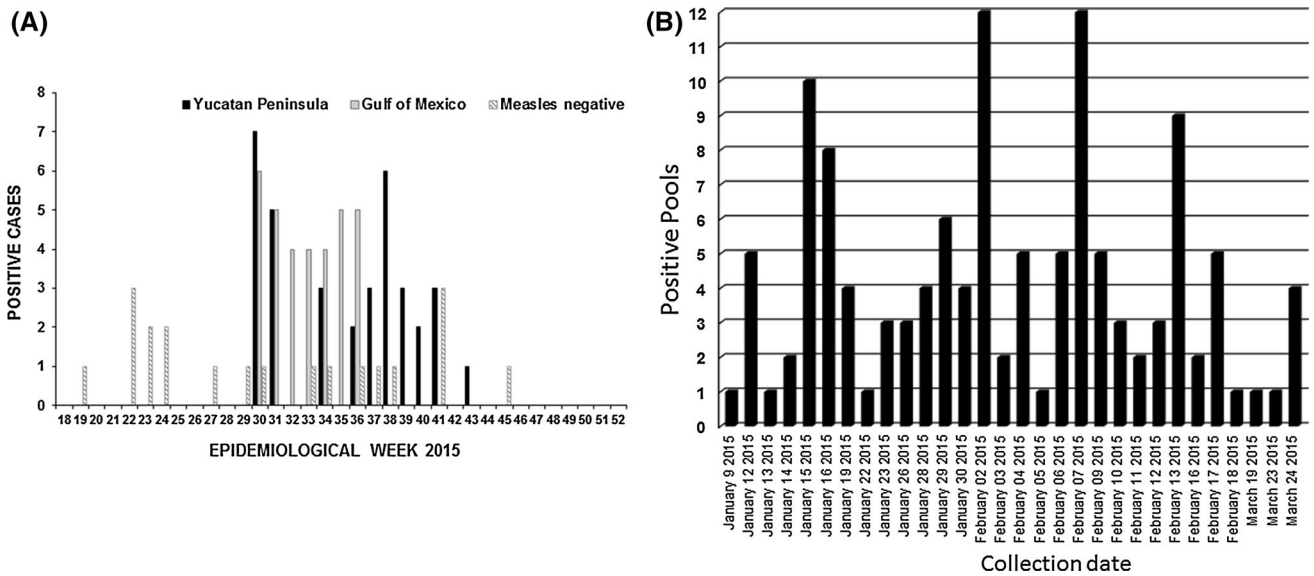

Fig. 1 **a** ZIKV RT-PCR analyses of 100 samples that were collected by the RNLSP from the Gulf of Mexico (Veracruz) and the Yucatán Peninsula but were negative for CHIKV or DENV as well as 21 samples that were non-confirmed suspected measles cases collected in 11 different Mexican states. A number of ZIKV-positive cases are shown, indicating the epidemiological week of the symptoms onset. **b** ZIKV RT-PCR analyses of *A. aegypti* mosquito pools that were collected in Guerrero state (South east Mexico) during the January–March 2015 period. The collection date (*x axis*) and the number of positive cases (*y axis*) are indicated

vector was already carrying the virus at the very beginning of 2015 (Fig. 1b). Interestingly, despite the fact that the Mexican Epidemiological Surveillance System has implemented protocols for microcephaly surveillance, no cases have been reported in these states yet, probably due to the fact that we have not reached a considerable burden of disease as previously reported by Brasil et al. [7]. In addition, no increase in Guillain–Barré syndrome or other child born defects linked to the ZIKV have been described.

This study highlights firstly the usefulness of retrospective analyses of available samples to establish the scope of the Zika virus epidemic, and secondly the potential need to revise and update the diagnostic algorithms of flavivirus and viral exanthems in endemic areas with state-of-the-art available technology.

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Author's contributions José Alberto Díaz-Quiñonez designed the experimental strategies; reviewed the results, figures, and text; contributed to fruitful discussions; and wrote the manuscript. Irma López-Martínez designed the experimental strategies, reviewed the results, and contributed to fruitful discussions. Belem Torres-Longoria designed the experiments, reviewed the results, and contributed to discussions. Mauricio Vázquez-Pichardo designed and performed the experiments, and constructed the figure. Edith Cruz-Ramírez reviewed the results and contributed to discussions. José Ernesto Ramírez-González carried out sequencing of PCR fragments and DNA data analyses. Cuitláhuac Ruiz-Matus designed the experimental strategies, reviewed the results, and contributed to fruitful discussions. Pablo Kuri-Morales designed the experimental strategies, reviewed the results, and contributed to fruitful discussions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest whatsoever.

Ethical approval This article uses human samples as part of the Mexican Epidemiological Surveillance System, according to the Mexican law (*NOM-017-SSA2-2012 para la vigilancia epidemiológica*).

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