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# Preliminary study does not demonstrate SARS-CoV-2 infection in bats from Oaxaca, México during the Covid-19 pandemic

# Estudio preliminar no demuestra infección por SARS-CoV-2 en murciélagos en Oaxaca, México durante la pandemia de Covid-19

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The SARS-CoV-2 virus is the etiological agent of the 2019 coronavirus disease that caused the COVID-19 pandemic. This virus has achieved sustained transmission across human populations; however, since 2020, reverse zoonotic contagion events have been documented, *i.e.*, the transmission of the virus from humans to domestic and wild animals. This study aims to determine the natural infection with SARS-CoV-2 coronavirus in bats captured in Oaxaca, México. Between October 2021 and February 2022, liver samples from 6 bat species were collected to detect SARS-CoV-2 RNA by quantitative Real-Time Polymerase Chain Reaction assay. No natural infection was detected in the samples tested, and no evidence of SARS-CoV-2 infection was found in the bats examined. It is necessary to test other organs (*e.g.,* trachea, lymph nodes, lung, heart, and kidney), biological samples (*e.g.*, saliva, urine, and nasal secretions), and a larger number of samples to clarify whether bats in Oaxaca are susceptible to infection with the SARS-CoV-2 coronavirus.

**Key words:** Chiroptera; coronavirus; virus; zoonosis.

El virus SARS-CoV-2 es el agente etiológico de la enfermedad por coronavirus de 2019 que provocó la pandemia de Covid-19. Este virus ha logrado una transmisión sostenida entre poblaciones humanas; sin embargo, desde 2020 se han documentado eventos de contagio zoonótico inverso, es decir, la transmisión del virus de humanos a animales domésticos y salvajes. Este estudio tiene como objetivo determinar la infección natural por el coronavirus SARS-CoV-2 en murciélagos capturados en Oaxaca, México. Entre octubre de 2021 y febrero de 2022, se recolectaron muestras de hígado de 6 especies de murciélagos para detectar el ARN del SARS-CoV-2 mediante el ensayo cuantitativo de Reacción en Cadena de la Polimerasa en tiempo real. No se detectó infección natural en ninguna de las muestras analizadas y no se encontró evidencia de infección por SARS-CoV-2 en los murciélagos examinados. Es necesario analizar otros órganos (*e.g.*, tráquea, ganglios linfáticos, pulmón, corazón y riñón), muestras biológicas (*e.g.*, saliva, orina y secreciones nasales) y un mayor número de muestras para aclarar si los murciélagos en Oaxaca son susceptibles a la infección por el coronavirus SARS-CoV-2.

**Palabras clave:** Coronavirus; quirópteros; virus; zoonosis.

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The SARS-CoV-2 virus is the etiological agent of the 2019 coronavirus disease that has caused the current COVID-19 pandemic. This virus emerged in late December 2019, and since then more than 676 million cases have been documented in humans, which caused the death of over 6 million people worldwide (World Health Organization, WHO 2020). The SARS-CoV-2 virus has achieved widespread and sustained human-to-human transmission in human populations. However, there is the potential of reverse zoonotic events, *i.e.*, the transmission of SARS-CoV-2 from humans to domestic and wild animals, which, in turn, may spread the disease among their conspecifics (Meekins *et al.* 2021; Kwon *et al.* 2023). This may impact domestic and wild animal populations, may affect new reservoir species, can cause the emergence of new mutant virus strains, and the risk of secondary infection for humans (Fischhoff *et al.* 2021; Meekins *et al.* 2021). For example, it is known that in Denmark and the Netherlands, SARS-CoV-2 was transmitted from humans to farm minks (*Neovison vison*) and then retransmitted from minks to humans again (Oude-Munnink *et al.* 2021).

Reverse zoonosis events of SARS-CoV-2 have been reported in companion animals such as cats (*Felis catus*), dogs (*Canis lupus familiaris*), hamsters (*Phodopus campbelli*); farm animals such as pigs (*Sus scrofa domestica*) and cows (*Bos taurus*); wild and zoo animals such as white-tailed deer (*Odocoileus virginianus*), dwarf otters (*Aonyx cinereus*), tigers (*Panthera tigris*), lions (*Panthera leo*), and gorillas (*Gorilla gorilla*; Fischhoff *et al.* 2021; Meekins *et al.* 2021; Kwon *et al.* 2023; Li *et al.* 2023). Experimental and modeling studies have shown that a wide variety of animal species are susceptible to SARS-CoV-2 infection, including non-human primates (*Macaca mulatta*, *M. fascicularis*, *Chlorocebus aethiops*, *Papio hamadryas*, *Callithrix jacchus*), shrews (*Tupaia belangeri*), rodents (*Peromyscus maniculatus*), and bats (*Rhinolophus thomasi*, *R. rex*, *Rousettus aegyptyacus*; Fischhoff *et al.* 2021; Meekins *et al.* 2021; Li *et al.* 2023).

Bats have a unique and sophisticated immune system that allows them to tolerate viral infections that are fatal in humans, such as Ebola and Marburg (Weinberg and Yovel 2022). Anthropogenic landscape modifications (*i.e.,* deforestation, urbanization, and agriculture) have led some bat species to adapt and use the new resources available (*i.e.,* alternative food and roosts) in the transformed areas. For instance, some frugivorous and insectivorous bat species of the genera *Artibeus, Carollia*, *Eptesicus*, *Eumops, Glossophaga*, *Lasiurus*, *Molossus,* and *Tadarida* use parks, crops, fruit trees, water bodies, and lighting sources within human settlements to feed, as well as abandoned houses for shelter; therefore, they may be exposed to SARS-CoV-2 from infected humans (Hall *et al.* 2023). The increasingly frequent interaction between wildlife and humans highlights the importance of the implementation and correct use of biosecurity measures to protect wildlife managers, the general population, and wild animals (Aguilar-Setién *et al.* 2022).

From 2019 to 2022, we carried out a study that aimed to determine the natural arbovirus infection in bats captured in caves, forests, and human settlements in the State of Oaxaca, México. Since some samples of bats captured in human settlements and the periphery of the urbanized area were available, they were tested for SARS-CoV-2 RNA. Therefore, this study aimed to determine the potential infection with the SARS-CoV-2 coronavirus in bats caught in Oaxaca, México.

The study was carried out in 2 municipalities of the State of Oaxaca: San Miguel Lachiguiri (hereinafter Lachiguiri), a town in the municipality of Santiago Lachiguiri with 500 inhabitants, and Santa Maria Huatulco (hereinafter Huatulco) a town of 50,862 inhabitants (INEGI 2020; Figure 1). Huatulco is a tourist destination that receives around 730,000 tourists annually (93 % national and 6.9 % foreign tourists). The number of confirmed cases of Covid-19 in 2022, when the present study was carried out, was 2 for

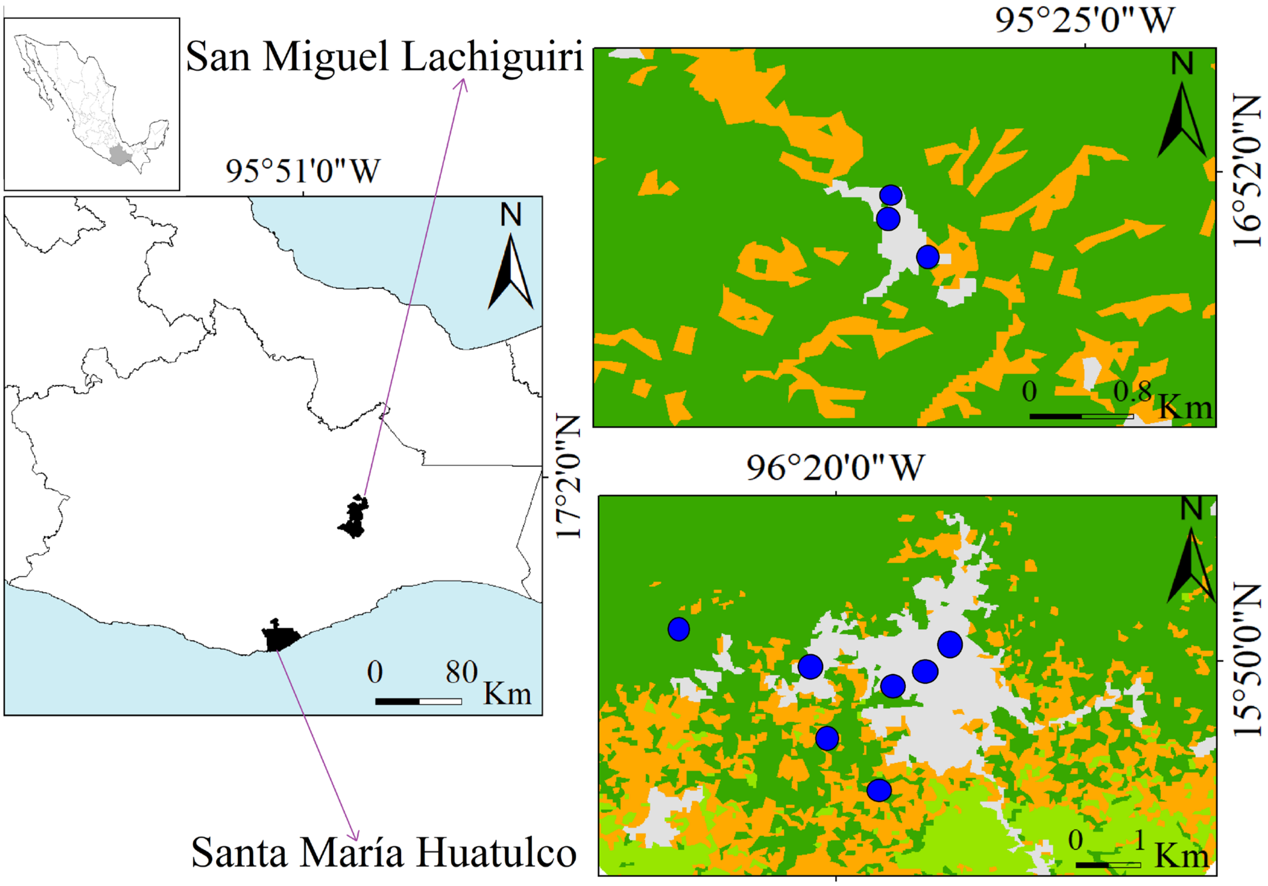

**Figure 1**. Study area and bat collection sites in San Miguel Lachiguiri and Santa María Huatulco, Oaxaca, México. The blue circles indicate the sampling sites where bats were captured. The white polygons on the right indicate the urban area; yellow, crops and pastures; and green, medium semi-evergreen forests.

the municipality of Santiago Lachiguiri and 2,018 for Santa María Huatulco (Dirección General de Epidemiología 2024).

Bats were captured (with SEMARNAT collection license FAUT-0143 from C. Lorenzo) monthly at 10 sites (7 in Huatulco and 3 in Lachiguiri) within the urban area and periphery of Lachiguiri and Huatulco, between October 2021 and February 2022 (Figure 1). The sites included the courtyards of 4 houses, a school, a church, an ornamental plant nursery, a pig farm, a secondary vegetation site located on the periphery of the urban area, and a medium semi-evergreen forest patch (Figure 2). All sites, except those with vegetation, were permanently used by people throughout the sampling period. Bats were captured with 3 mist nets, which remained open from 18:00 hr to 23:30 hr. To reduce the risk of human-to-wildlife transmission all the staff had received at least 1 dose of a SARS-CoV-2 vaccine before field trips. Additionally, although only a single person was designated to handle bats during field sampling, all staff performed hand disinfection with antibacterial gel and 70 % ethyl alcohol and used N95 face masks and nitrile gloves. Each bat captured was weighed, and the standard external somatic metrics were recorded. Of the total number of bats captured at the 10 sites, 79.6 % were released at the capture site (*n* = 78) without taking any samples for virus diagnosis; 20.4 % (*n* = 20) were sedated with 2 % isoflurane inhaled for 2 min and then euthanized with an intracardiac injection of 0.2 mL of sodium pentobarbital. Because the main objective of the study was the search for arboviruses in bats, the project only considered liver samples and it was not possible to collect samples from other organs where coronaviruses have been detected in bats (*i.e.*, lungs, heart, kidneys; Schlottau *et al.* 2020; Li *et al.* 2023). Liver samples were extracted and stored in cryogenic vials (Corning Incorporated, Corning, N.Y., U.S.) containing 1 mL of RNA*later*TM (Sigma-Aldrich Chemical) stabilizing solution. All bat specimens are deposited in the Mammal Collection of El Cole-

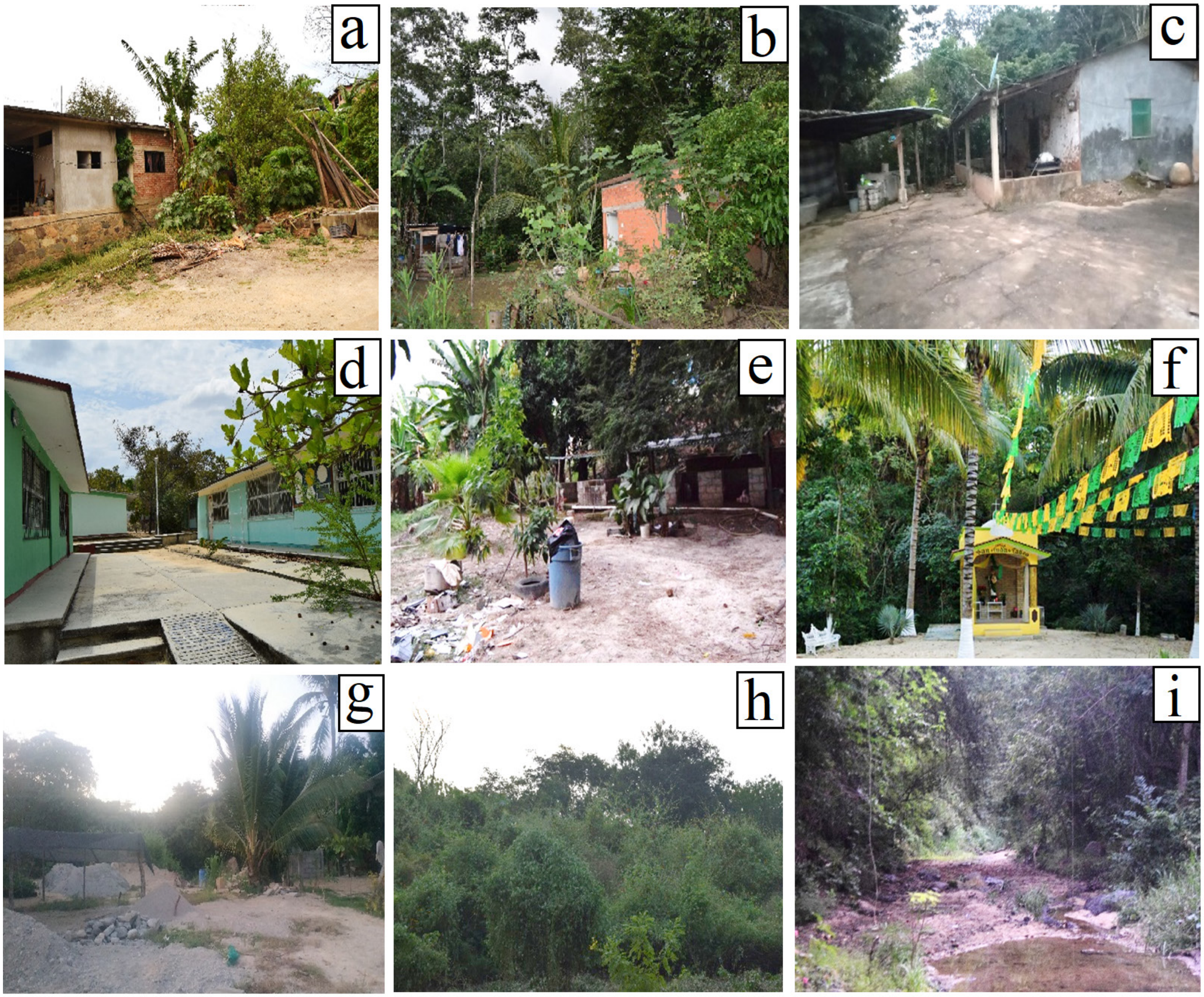

**Figure 2**. Representative photographs of the bat sample collection sites in San Miguel Lachiguiri and Santa María Huatulco, Oaxaca, Mexico. a-c) sampling sites within human settlements in San Miguel Lachiguiri; d) school; e) pig farm; f) church; g) plant nursery; and h-i) vegetation in Santa María Huatulco. Photographs: I. Hernández Aguilar.

gio de la Frontera Sur, San Cristóbal de Las Casas, Chiapas, México.

We used the Berlin qRT-PCR protocol for detecting gene E (Envelope Protein Gene) of sarbecoviruses SARS, SARS-CoV-2 as the screening test and the RDRP gene as a confirmatory test, as needed. The reaction mixture for the gene E included 5 µl of RNA, 12.5 µl of 2X buffer of the Superscript™ III Platinum® One-Step system, 4.1 µl of molecular biology-grade water (Invitogren), 0.4 µl of $MgSO_4$ (50 mM), 0.5 µl of Superscript™ III Platinum® One-Step enzyme, 1 µl of primers (10 µm), and 0.5 µl of probe (10 µM). A probe reactive to SARS-CoV (P2) was used (Corman *et al.* 2020). The primers and probe used were manufactured by T4 Oligo (Guanajuato, México) with the following sequences (5´- 3´): E Sarbeco F1 primer (ACA GGT ACG TTA ATA GTT AAT AGC GT), E Sarbeco R2 primer (ATA TTGCAG CAGTACGCACACA), and the E-Sarbeco P1 probe (FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-BHQ1). The qRT-PCR protocol was performed on an ABI FAST 7500 (Applied Biosystems) thermal cycler under the following conditions: reverse transcription (RT) at 50 °C for 15 min, followed by 1 cycle at 95 °C for 2 min, and, finally, 45 cycles at 95 °C for 15 sec and 60 °C for 30 sec (where fluorescence detection was performed). We used a positive control donated by the Instituto de Diagnóstico y Referencia Epidemiológicos (Institute of Epidemiological Diagnosis and Reference; InDRE, México) and molecular biology-grade water (Qiagen, México) as a negative control. A sample was deemed positive when fluorescence exceeded the Cycle Threshold of 22 and yielded a sigmoidal amplification curve (Figure 3a). The analyses were carried out in the Virology and Molecular Biology Laboratory of the Laboratorio Estatal de Salud Pública de Oaxaca (LESPO) in Oaxaca City, México.

A total of 20 liver samples from 6 bat species of the family Phyllostomidae were analyzed: the frugivores *Artibeus lituratus* (45 % of the samples analyzed), *A. jamaicensis* (25 %), *A. toltecus* (10 %), and *Carollia perspicillata* (5 %), the nectarivore *Glossophaga mutica* (10 %), and the hematophagous bat *Desmodus rotundus* (10 %). Of the bat specimens collected, 15 were males (73.3 % with scrotal tested and 26.7 % non-reproductive) and 5 were females (all with no signs of reproductive evidence). Of all samples, 60 % were collected at Huatulco (including 1 medium semi-evergreen forest site, 1 secondary vegetation site, 1 plant nursery, 1 pig farm, 1 school, 1 church, and the yard of 1 house), and 40 % at Lachiguiri (in the yard of 3 houses; Table 1). All liver samples analyzed by qRT-PCR tested negative for SARS-CoV-2 RNA (Figure 3b).

Other coronaviruses that cause serious and deadly respiratory diseases, such as MERS-CoV and SARS-CoV, are generalist viruses that are not unique to humans and they can cross species barriers and adapt to new host species, so they infect a wide range of animals, such as pigs, cows, birds, cats, ferrets, rabbits, rodents, and bats (Chan *et al.* 2013; Cui *et al.* 2019; Li *et al.* 2023). The SARS-CoV-2 virus also has the ability to spread to multiple species and establish natural reservoirs, including domestic and wild animals (Kwon *et al.* 2023; Li *et al.* 2023). The wide range of coronavirus hosts, including SARS-CoV-2, is partly due to their use of the angiotensin-converting enzyme 2 (ACE2) receptor to enter host cells; this receptor is present in major vertebrate groups, such as mammals, birds, reptiles, and amphibians (Chou *et al.* 2006; Letko *et al.* 2020).

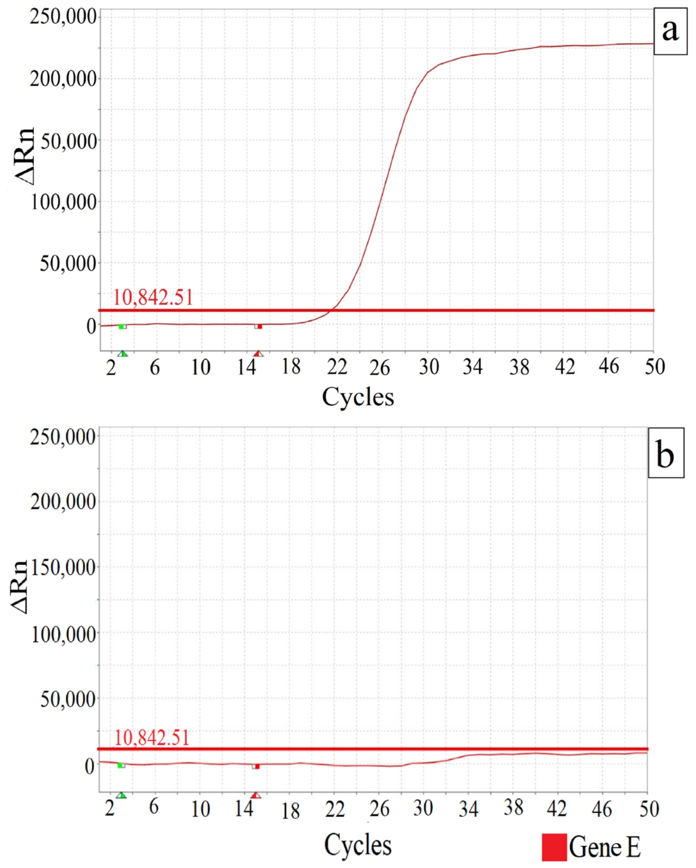

**Figure 3.** Plots of qRT-PCR (quantitative real-time polymerase chain reaction) amplification for a) the positive and negative controls and b) an unamplified sample for SARS-CoV-2 RNA, in bats captured at Santa María Huatulco and San Miguel Lachiguiri, Oaxaca, México.

The documented mode of transmission of SARS-CoV-2 in animals (cats, mink, and bats) is direct contact transmission through respiratory microdroplets or aerosols (Bosco-Lauth *et al.* 2021; Shi *et al.* 2020). In bats, SARS-CoV-2 has been detected in nasal turbinates, trachea, lymph nodes, lungs, heart, adrenal gland, kidneys, and duodenum of *Rhinolophus thomasi, R. rex,* and *Roussettus aegyptiacus* (Schlottau *et al.* 2020; Li *et al.* 2023). A recent review indicated that coronaviruses have been detected more frequently in the feces and gut of bats in the Americas (Hernández-Aguilar *et al.* 2021). Therefore, future studies in bats should include samples of nasal secretions, oral fluids, urine, feces, or other tissues where SARS-CoV-2 has been detected previously.

Studies of SARS-CoV-2 reverse zoonoses have been focused on domestic and companion animals, probably due to their greater proximity and frequency of interaction with humans. In bats, studies have been carried out mainly of experimental infection. For example, Hall *et*

**Table 1**. Characteristics of samples tested for SARS-CoV-2 viral RNA from bats captured at Santa Maria Huatulco and San Miguel Lachiguiri, Oaxaca, México. M: Male; F: Female; CR: Reproductive condition; ST: Scrotal testes; NR: Non-reproductive.

| Species | Sex | CR | Collection Date | Municipality | Collection site |
|---|---|---|---|---|---|
| *Artibeus jamaicensis* | M | ST | 24/10/2021 | Lachiguiri | Yard of a house |
| *Artibeus jamaicensis* | F | NR | 24/10/2021 | Lachiguiri | Yard of a house |
| *Artibeus jamaicensis* | M | ST | 25/11/2021 | Huatulco | Plant nursery |
| *Artibeus jamaicensis* | M | NR | 26/11/2021 | Huatulco | School |
| *Artibeus jamaicensis* | F | NR | 27/01/2022 | Huatulco | Yard of a house |
| *Artibeus lituratus* | F | NR | 24/10/2021 | Lachiguiri | Yard of a house |
| *Artibeus lituratus* | M | ST | 26/11/2021 | Huatulco | School |
| *Artibeus lituratus* | M | ST | 26/11/2021 | Huatulco | School |
| *Artibeus lituratus* | M | ST | 27/11/2021 | Huatulco | School |
| *Artibeus lituratus* | M | ST | 15/12/2021 | Lachiguiri | Yard of a house |
| *Artibeus lituratus* | M | ST | 27/01/2022 | Huatulco | Yard of a house |
| *Artibeus lituratus* | M | ST | 30/01/2022 | Huatulco | Pig farm |
| *Artibeus lituratus* | M | ST | 30/01/2022 | Huatulco | Pig farm |
| *Artibeus lituratus* | F | NR | 09/02/2022 | Lachiguiri | Yard of a house |
| *Artibeus toltecus* | M | NR | 27/01/2022 | Lachiguiri | Yard of a house |
| *Artibeus toltecus* | M | ST | 09/02/2022 | Lachiguiri | Yard of a house |
| *Carollia perspicillata* | M | NR | 24/11/2021 | Huatulco | Church |
| *Desmodus rotundus* | F | NR | 30/01/2022 | Huatulco | Vegetation |
| *Glossophaga mutica* | M | NR | 24/10/2021 | Lachiguiri | Yard of a house |
| *Glossophaga mutica* | M | ST | 15/12/2021 | Huatulco | Vegetation |

al. (2021) inoculated SARS-CoV-2 in *Eptesicus fuscus* and did not detect viral RNA in oral and oropharyngeal secretions, in addition to no transmission to uninoculated bats. Schlottau *et al.* (2020) and Hall *et al.* (2023) also inoculated SARS-CoV-2 in *R. aegyptiacus* and *Tadarida brasiliensis* and found that although these species can maintain active SARS-CoV-2 infection, they do not present clinical signs of the disease. Li *et al.* (2023) included kidney, pancreas, heart, and brain samples from 26 species of bats from the families Vespertilionidae, Emballonuridae, Hipposideridae, Molossidae, Pteropodidae, and Rhinolophidae but only found positive results in *R. thomasi* and *R. rex*. Gultom *et al.* (2021) analyzed airway epithelial cells of *Sturnira lilium* and *Carollia perspicillata* and found that they are not susceptible to SARS-CoV-2. Moran *et al.* (2023) collected 235 saliva samples from lactating females of *Myotis lucifugus* in the United States, and none tested positive for SARS-CoV-2. This indicates that some bat species are susceptible to SARS-CoV-2 infection, and although bat-to-bat transmission is possible, it is inefficient (Meekins *et al.* 2021). Despite this, so far, most bat species have not been considered in natural or experimental infection studies. However, transmission of SARS-CoV-2 from bats or humans to other animal species is a latent possibility that requires further investigation. Therefore, more research is needed to help understand the susceptibility of bats to SARS-CoV-2.

In addition, because some species of tropical bats, such as *Artibeus jamaicensis*, *A. lituratus*, *Carollia* sp. and *Glossophaga* sp., are frequently associated with human households and can interact with humans, farm animals, and pets, bats should be included in any wildlife monitoring program against SARS-CoV-2. It is estimated that for everyone thousand individuals of *Myotis lucifugus,* between 0.47 and 1.56 individuals may be potentially infected when manipulated by SARS-CoV-2-infected humans (Cook *et al.* 2022). However, personal protective equipment, such as face masks, or a negative COVID-19 test before coming into contact with bats can reduce the transmission risk by between 65 % and 88 % (Cook *et al.* 2022). Therefore, we recommend that, as far as possible, people coming into contact with live bats use the biosecurity measures recommended by Aguilar-Setién *et al.* (2022) for their protection and to prevent the transmission of SARS-CoV-2. In addition to those used in this study, the use of nitrile gloves, a Tyvek suit, washable footwear (plastic boots) or footwear covers, and clear glasses with full coverage to the eye area are recommended, in addition to a complete vaccination scheme.

The search for wild reservoirs of SARS-CoV-2 is essential to understand the ecology of the disease and anticipate future complications of the pandemic, establish standardized preclinical models for the development and efficacy testing of vaccines and treatments against SARS-CoV-2, and foresee future zoonotic scenarios (Meekins *et al.* 2021; Li *et al.* 2023).

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