Virology Journal

RESEARCH Open Access

# Detection of avian, murine, bovine, shrew, and bat coronaviruses in wild mammals from Mexico

Jocelyn Medina-Gudiño[1], Yolanda López-Vidal[2], J. Adolfo Pardo-Tovar[1], Lauro Velázquez-Salinas[3] and Francisco Javier Basurto-Alcántara[1*]

## Abstract

Coronaviruses infect a wide range of animal and human hosts. Some human coronaviruses, such as SARS-CoV, MERS-CoV, and SARS-CoV-2, originated in animals, with bats often serving as ancestral hosts. This study analyzed samples from wild animals in three Mexican states, using an RT-PCR assay targeting the *RdRp* gene to detect and genotype coronaviruses, assessing their potential role as reservoirs. Phylogenetic analysis was conducted to determine the genetic relationships of the identified coronaviruses. *Gammacoronavirus* RNA was identified in fallow deer, llamas, spider monkeys, and mouflons; *Betacoronavirus* RNA in mouflons and dwarf goats; and *Alphacoronavirus* RNA in dwarf goats and ponies. The detected viral sequences exhibited high nucleotide identity with known coronaviruses, including *Avian coronavirus* (*Gammacoronavirus*), *Murine coronavirus* (*Betacoronavirus*), *Betacoronavirus 1* (*Betacoronavirus*), Wénchéng shrew coronavirus (unclassified *Alphacoronavirus*), and *Bat coronavirus HKU10* (*Alphacoronavirus*). These findings represent the first report of *Avian coronavirus*, *Murine coronavirus*, Wénchéng shrew coronavirus, and *Bat coronavirus HKU10* in these species, as well as the first detection of *Avian coronavirus* in llamas, spider monkeys, and mouflons. This study provides valuable insights into the potential role of wildlife as coronavirus reservoirs, highlighting the importance of monitoring these viruses to mitigate future zoonotic transmission risks.

**Keywords** Coronavirus reservoirs, Coronavirus in wildlife, *Gammacoronavirus*, *Betacoronavirus*, *Alphacoronavirus*

## Introduction

Coronaviruses are enveloped, single-stranded, positive-sense RNA viruses. They belong to the family *Coronaviridae* (subfamily *Orthocoronavirinae*, order *Nidovirales*), which consists of four genera: *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus*, and *Gammacoronavirus* [1]. Coronaviruses can infect a wide range of species, including bats, camels, ferrets, mink, pigs, cattle, birds, and humans [2], and they can cause diverse neurological, respiratory, digestive, and reproductive signs [1].

Mutation and adaptation have shaped the coevolution of coronaviruses and their hosts over time. An evolutionary host harbors an ancestral virus, typically in an apathogenic form, while a reservoir can maintain the virus long-term. When a virus is introduced to an intermediate host, it may cause a dead-end infection if it can't sustain transmission, or the host may act as an amplifier, temporarily replicating the virus before passing it to another host, such as a human. If adaptation occurs, the virus may become pathogenic or even establish long-term

*Correspondence:
Francisco Javier Basurto-Alcántara
basurto@unam.mx
[1] Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad de Mexico, México
[2] Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de Mexico, México
[3] Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, United States Department of Agriculture-Agricultural Research Service, Greenport, NY, USA

endemicity in the intermediate host, turning it into a new natural reservoir [3].

As demonstrated in the emergence of SARS-CoV-2, coronaviruses are the main component of an intricate epidemiological puzzle involving human habits and viral biological mechanisms [4]. Transmission of viruses from animals to humans or other animals has occurred in the past but has increased in frequency in recent decades [5]. The intensification of climate change in the first quarter of the twenty-first century has favored the spread of diseases due to the modification of ecological niches [5]. In addition, deforestation and urbanization promote spillover by reducing the habitat of wild animals and increasing the contact rate between animals of different species and humans [4]. Meanwhile, contact between wild animals and humans in places such as food markets represents favorable scenarios for spillover, adaptation to new hosts, and, eventually, species jumping [6].

Most human coronaviruses (HCoVs) originate from domestic and wild animal reservoirs [7]. Studies that infer the evolutionary history of these viruses suggest that SARS-CoV, MERS-CoV, SARS-CoV-2, HCoV_NL63, and HCoV_229E have their origins in bat viruses, with intermediate hosts being masked palm civets, camelids, and alpacas, respectively [8, 9]. On the other hand, HCoV_OC43 and HCoV_HKU1 are thought to have originated from rodent-ancestral viruses [6]. Although HCoV_OC43 can infect camels, it probably had bovines as intermediate hosts [10].

Human coronaviruses, such as NL63, 229E, OC43, and HKU1, cause the common cold and do not need animal reservoirs. On the other hand, the highly pathogenic SARS-CoV and MERS-CoV have not fully adapted to humans and cannot sustain transmission within human populations. As a result, they are likely to spread to susceptible humans through intermediate hosts [3], as seen in the case of camels for MERS-CoV [9].

Coronaviruses can infect animals beyond their common hosts. For instance, researchers have detected canine coronavirus in humans in Malaysia [11], HCoV_OC43 in pigs and dogs [6], and SARS-CoV-2 in various animals, including cats, dogs, mink, tigers, and deer [12–14]. Additionally, experimental studies have demonstrated the ability of coronaviruses to infect cells derived from species other than their natural hosts. For example, although no natural infections of mammals with the avian infectious bronchitis virus have been reported, the virus can adapt to mammalian cell lines, including Vero (green monkey), BHK-21 (golden hamster), HEK-293 T (human), and HeLa (human) [15].

Human-animal interactions can have beneficial effects by promoting pro-conservation behaviors in humans; however, they also pose ethical and animal welfare risks that must be carefully managed [16, 17]. Animals under human care are those maintained and protected in controlled environments such as zoos, aquariums, and conservation centers (known in Mexico as animal management units). These facilities must adopt a comprehensive approach that balances individual well-being with species conservation goals, utilizing ethical frameworks such as the duty of care, compassionate conservation, and conservation welfare [16, 18, 19]. The term "animals under human care" conveys both an ethical and practical responsibility to evaluate and safeguard the physical and psychological well-being of these animals through behavioral, physiological, and clinical indicators, as well as frameworks such as the five domains model [20–23].

Animals in zoos and conservation centers can serve as sentinels for viral disease surveillance, providing valuable data on the circulation of infectious agents in animal populations that are difficult to study in the wild [24]. Detecting infected individuals facilitates the implementation of targeted management measures to protect susceptible animals and minimize the risk of pathogen transmission [25]. Identifying reservoirs, natural hosts, and intermediate hosts plays a crucial role in disease prevention. In Mexico, information on the genetic diversity of coronaviruses, their hosts, and reservoirs remains limited [26, 27].

Considering the relevance of reservoirs and intermediate hosts in the evolution of coronaviruses, this study aimed to detect and genotype coronaviruses in wild animals in Mexico to determine their frequency in these populations and identify potential wild reservoirs of coronaviruses. For this purpose, samples of feces, rectal, nasal, oral, and cloacal swabs were obtained from wild animals under human care from three Mexican states. Total RNA was extracted from the samples to amplify a conserved coronavirus *RdRp* gene (*nsp12*) region through RT-PCR. The products were sequenced and analyzed to infer their phylogenetic relationship.

## Materials and methods

### Sampling

Feces, rectal/cloacal, oral, and nasal swabs were collected from 140 clinically healthy wild animals of 43 species/subspecies under human care across three states in Mexico: the State of Mexico, Tamaulipas, and Mexico City. The sampled animals included: mandarin duck, yellow-headed amazon, Aoudad, northern pintail, spider monkey, axis deer, ring-tailed cat, coyote, gray wolf, Mexican wolf, Canadian wolf, dwarf goat, elk, red deer, wildebeest, hyena, fallow deer, black-bellied tree duck, emu, pony, zebra, Aztec parrot, llama, lemur, gray fox, Japanese macaque, budgerigar, coati, mouflon, chimpanzee, lion, white lion, barbary lion, jaguar, leopard, tiger,

Bengal tiger, sacred pochard, Harris's hawk, peacock, blue-headed parrot, raccoon, and cougar (Table 1). The samples were stored at − 70 °C until processing.

### Strains

Avian infectious bronchitis virus (AIBV) vaccine strain Ma5 (*Gammacoronavirus*) and Canine coronavirus (CCoV) vaccine strain 1–71 (*Alphacoronavirus*) were propagated in 10-day-old SPF chicken embryos and A-72 cells, respectively. Both strains were used as controls in RT-PCR assays.

### Plasmids

Three plasmids were designed for use as controls in PCR assays. These synthetic plasmids were acquired from Integrated DNA Technologies, Inc. The pBENT vector contains a 743 bp fragment of the bovine coronavirus (*Betacoronavirus) RdRp* gene. The pPHKU vector includes a 255 bp fragment of the porcine coronavirus HKU15 (*Deltacoronavirus*) *RdRp* gene, and the pTHKU vector contains a 473 bp fragment of the thrush coronavirus HKU12 (*Deltacoronavirus*) *RdRp* gene.

### Sequences

All complete and curated assembly reference sequences of the *Coronaviridae* family (updated as of April 3, 2023) were downloaded from the NCBI Virus database for primer design and subsequently used in phylogenetic analysis.

### Primers

Reference coronavirus sequences were imported into Benchling® software, and the *nsp12* gene was manually identified within the ORF1b of each sequence based on (1) a comparison of the inferred amino acid sequences from each reading frame with the amino acid sequences of coronavirus nsp12 available in UniProt, and (2) a multiple sequence alignment of sequences from members of each coronavirus genus, performed using Clustal Omega. To identify conserved regions potentially suitable for the design of *nsp12*-targeted primers, multiple sequence alignments of various subsets of each genus were performed using MAFFT (local pairwise). After identifying conserved *nsp12* regions, primers were manually designed according to standard criteria, targeting annealing temperatures near 65 °C for all primer pairs. Due to the higher sequence diversity observed in *nsp12* among *Betacoronavirus* and *Deltacoronavirus* members,

**Table 1** Scientific name, common name, and number of sampled individuals

| Scientific name | Common name | Number of individuals |
|---|---|---|
| *Aix galericulata* | Mandarin duck | 3 |
| *Amazona oratrix* | Yellow-headed amazon | 1 |
| *Ammotragus lervia* | Aoudad | 2 |
| *Anas acuta* | Northern pintail | 2 |
| *Ateles geoffroyi vellerosus* | Spider monkey | 1 |
| *Axis axis* | Axis deer | 8 |
| *Bassariscus astutus* | Ring-tailed cat | 2 |
| *Canis latrans* | Coyote | 3 |
| *Canis lupus* | Gray wolf | 3 |
| *Canis lupus baileyi* | Mexican wolf | 4 |
| *Canis lupus occidentalis* | Canadian wolf | 3 |
| *Capra aegagrus hircus* | Dwarf goat | 26 |
| *Cervus canadensis* | Elk | 1 |
| *Cervus elaphus* | Red deer | 1 |
| *Connochaetes taurinus* | Wildebeest | 1 |
| *Crocuta crocuta* | Hyena | 1 |
| *Dama dama* | Fallow deer | 11 |
| *Dendrocygna autumnalis* | Black-bellied tree duck | 3 |
| *Dromaius novaehollandiae* | Emu | 2 |
| *Equus ferus caballus* | Pony | 1 |
| *Equus zebra* | Zebra | 1 |
| *Eupsittula canicularis* | Aztec parrot | 1 |
| *Lama glama* | Llama | 2 |
| *Lemur catta* | Lemur | 1 |
| *Lycalopex griseus* | Gray fox | 3 |
| *Macaca fuscata* | Japanese macaque | 1 |
| *Melopsittacus undulatus* | Budgerigar | 1 |
| *Nasua nasua* | Coati | 6 |
| *Ovis aries musimon* | Mouflon | 16 |
| *Pan troglodytes* | Chimpanzee | 2 |
| *Panthera leo* | Lion | 2 |
| *Panthera leo krugeri* | White lion | 2 |
| *Panthera leo leo* | Barbary lion | 2 |
| *Panthera onca* | Jaguar | 4 |
| *Panthera pardus* | Leopard | 2 |
| *Panthera tigris* | Tiger | 2 |
| *Panthera tigris tigris* | Bengal tiger | 5 |
| *Papio hamadryas* | Sacred pochard | 1 |
| *Parabuteo unicinctus* | Harris's hawk | 2 |
| *Pavo cristatus* | Peacock | 1 |
| *Pionus menstruus* | Blue-headed parrot | 1 |
| *Procyon lotor* | Raccoon | 3 |
| *Puma concolor* | Cougar | 2 |
| Total count | | 140 |

two primer pairs were designed to improve the detection capacity for species within these genera.

### RNA extraction

Fecal, rectal, cloacal, oral, choanal, and nasal swab samples were diluted 1:10 in sterile PBS prior to RNA extraction. 0.1 $cm^3$ of feces were diluted in 900 µl of PBS and homogenized with a vortex mixer for 20 s. Rectal, oral and nasal swabs were placed in 2 ml microfuge tubes containing 1 ml of PBS, and swab heads were cut with sterile scissors. The swab samples were homogenized with a vortex mixer for 20 s and the swab heads were discarded. TRIzol™ Reagent was used for extracting total RNA from the samples, as well as strains Ma5 (AIBV) and 1–71 (CCoV). Briefly, 400 µl of the samples were mixed with 900 µl of TRIzol™ Reagent in sterile microcentrifuge tubes. The tube's content was mixed six times by upright inversion and incubated at 4 °C for 5 min. 240 µl of chloroform was added. The mixture was homogenized and incubated at 4 °C for 5 min. The tubes were centrifuged at 13,000 g and 4 °C for 15 min. 200 µl of the supernatant was transferred to a microcentrifuge tube and mixed with 600 µl isopropanol. The mixture was incubated at − 20 °C for 1 h and centrifuged at 13,000 g and 4 °C for 15 min. The supernatant was discarded, and the button was washed with 1 ml of 75% ethanol and centrifuged at 13,000 g and 4 °C for 5 min. The supernatant was discarded, and the sediment was dried at room temperature for 5 min. The buttons were suspended in 20 µl of nuclease-free water and stored at -70 °C until use.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Primers targeting the *nsp12* gene from all four genera within the *Orthocoronavirinae* subfamily were designed in this work (Table 2) and acquired from the Biotechnology Institute, UNAM. Reverse transcription was conducted following the manufacturer's recommendations for the M-MLV Reverse Transcriptase (Thermo-Fisher) in a 20 µl reaction volume. Briefly, 500 ng of RNA (1–10 µl), 0.2 µl of gene-specific primer (10 µM), 1 µl of dNTP Mix (10 mM each), and nuclease-free water (q.s. 12 µl) were combined and homogenized by pipetting, followed by a 5 min incubation at 65 °C. Then, 4 µl of First-Strand Buffer (5 X), 2 µl of DTT (0.1 M), and 1 µl of Ribonuclease Inhibitor (40 U/µl) were added, homogenized, and incubated for 2 min at 37 °C. After that, 1 µl of M-MLV Reverse Transcriptase (200 U/µl) was added and incubated for 50 min at 37 °C to synthesize the complementary DNA. The reaction was stopped by heating the mixture for 15 min at 70 °C.

The primers listed in Table 2 were utilized for the PCR amplification of the coronavirus *RdRp* gene target region. Briefly, 17.75 µl of nuclease-free water, 2.5 µl of DreamTaq Buffer (10X with 20 mM $MgCl_2$), 0.5 µl of dNTP mix (10 mM each), 0.25 µl of DreamTaq DNA polymerase (5 U/µl), 1 µl of primer F (10 µM), 1 µl of primer R (10 µM), and 2 µl of DNA (≤ 500 ng/ 2 µl) were combined and homogenized by pipetting.

Amplification was performed on a MiniAmp Plus thermal cycler. Initial denaturation was performed at 95 °C for 5 min, followed by 30 cycles with denaturation at 95 °C for 30 s, alignment at 65 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min.

### Sequencing

RT-PCR products were separated by electrophoresis on a 1% agarose gel, excised using a scalpel under UV transillumination, and purified following the manufacturer's instructions for the QIAquick® Gel Extraction Kit. The purified products were then sequenced using their respective forward and reverse primers on an ABI

**Table 2** Primers used for the RT-PCR amplification of the target region in the *RdRp* gene of orthocoronavirus

| Primer | Sequence | Length (nt) | Amplicon (bp) | Genus |
|---|---|---|---|---|
| Ac767 F | AGTGTGACCGTGCTTTACCT | 20 | 767 | *Alphacoronavirus* |
| Ac767 R | AGCAGGCTTAGGGTGTTTTGT | 21 | | |
| Bc338F | KCAGGATGGTAATGCTGCTATCA | 23 | 338 | *Betacoronavirus* |
| Bc338 R | TGCGRGCTCTATTCTTWGCAC | 21 | | |
| Bc743 F | TGTTTTGGGCCTCTTGTTAGG | 21 | 743 | *Betacoronavirus* |
| Bc743 R | TGAAACATTCTGCCAGTCATAGT | 23 | | |
| Dc255F | CTAAACAGTCAGTCAAGCCCG | 21 | 255 | *Deltacoronavirus* |
| Dc255 R | ACTGTGCATTAATACATCCACCT | 23 | | |
| Dc473 F | ACTATATGCAAGATGGTGAAGC | 22 | 473 | *Deltacoronavirus* |
| Dc473 R | GTCCCAACCACCATAAAATTTGG | 23 | | |
| Gc347F | TTATGGCGGTTGGGACAACA | 20 | 347 | *Gammacoronavirus* |
| Gc347 R | CACTCAAAAGACGCGCAACA | 20 | | |

PRISM® 3130xl Genetic Analyzer at the Biotechnology Institute, UNAM.

### Phylogenetic analysis

The obtained forward and reverse sequences were first aligned and subsequently assembled using MAFFT. Multiple sequence alignment was performed with Kalign, while phylogenetic reconstruction was conducted using the maximum likelihood method in IQ-TREE with 1,000 bootstrap replicates. The best-fitting model, determined according to the Bayesian Information Criterion (BIC), was the general time-reversible (GTR) nucleotide substitution model with Felsenstein's stationary nucleotide frequency estimates (F), rate heterogeneity among sites modeled with a proportion of invariant sites (I), and a gamma distribution with four rate categories (G4). The *RdRp* gene sequences of members from the subfamilies *Letovirinae* and *Pitovirinae* within the family *Coronaviridae* were used as outgroups. Highly similar sequences to the viral sequences detected in the samples analyzed by RT-PCR in this study were identified using BLAST. The FigTree software was used for visualizing the phylogenetic tree. Identity rates presented in Table 4 were obtained through pairwise comparison of the specified *nsp12* sequences using Clustal Omega.

### Viral isolation

To prepare the inoculum for 18-day-old SPF chicken embryos, PBS-eluted samples from swabs testing positive for *Gammacoronavirus* were filtered through bacteriological membranes (0.22 µm). Briefly, using an ovoscope and a pencil, the inoculation site was marked 5 mm above the air chamber, on the side opposite to the chicken embryo. The inoculation site was then disinfected with iodine, and the eggshell was pierced with an 18 G needle. Subsequently, 100 µl of the inoculum was deposited into the allantoic cavity using a 1-ml syringe with a 26 G needle. Finally, the inoculation site was sealed with white glue. Inoculated chicken embryos and non-inoculated controls were incubated for 48 h at 38 °C with a relative humidity of 80–90%. Euthanasia was performed by refrigerating the embryos at 4 °C for 24 h. Viral isolation was confirmed by observing the characteristic lesions of AIBV infection in chicken embryos and through RT-PCR from the allantoic fluid of inoculated chicken embryos, as well as from five successive serial passages.

## Results

*Gammacoronavirus* RNA was detected in the rectal swabs and feces from 6.3% of mouflons (1/16), 18.18% of fallow deer (2/11), 50% of llamas (1/2), and 100% of spider monkeys (1/1). *Betacoronavirus* RNA was detected in the rectal swabs and feces of 6.3% of mouflons (1/16) and 3.8% of dwarf goats (1/26). *Alphacoronavirus* RNA was detected in the rectal swabs and feces from 3.8% of dwarf goats (1/26) and in oral swabs from 100% of ponies (1/1). Positive samples for different viruses corresponded to different individuals (no coinfections were detected). A complete correlation was observed between the results of fecal samples and rectal swabs, as well as between nasal and oral swabs.

Sequencing of the RT-PCR products and phylogenetic reconstruction were conducted to identify the genus, species, and, if applicable, subspecies and strains of the coronavirus RNA detected in the samples from wild ruminants and primates from Mexico. The viral sequences detected in this study were submitted to GenBank under the accession numbers PQ243315-PQ243323 (Table 3), and their phylogenetic relationships within the *Coronaviridae* family are depicted in Fig. 1.

In the *RdRp* nucleotide sequences, identities greater than 99.4% were observed between the H120 strain of avian infectious bronchitis virus (AIBV, species *Avian coronavirus*, genus *Gammacoronavirus*) and the strains detected in one mouflon (99.42%), two fallow deer (99.71% and 100%), one llama (99.71%), and one spider monkey (97.98%). Additionally, the strain detected in one mouflon exhibited 98.98% identity with the A59 strain of murine hepatitis virus (MHV, species *Murine coronavirus*, genus *Betacoronavirus*), while the strain detected in one dwarf goat showed 99.73% identity with the E-AH65-TC strain of bovine coronavirus (BCoV, species *Betacoronavirus 1*, genus *Betacoronavirus*). Furthermore, the strain detected in one dwarf goat shared 98.99% identity with the Xingguo-74 isolate of Wénchéng shrew coronavirus (WESV, unclassified species, genus *Alphacoronavirus*), whereas the strain detected in one pony had 98.49% identity with the 183A isolate of Roussetus bat coronavirus HKU10 (HKU10, species *Bat coronavirus HKU10*, genus *Alphacoronavirus*). No *Deltacoronavirus* species were detected in any of the analyzed samples. The identities between the detected sequences and those of AIBV, MHV, BCoV, WESV, and HKU10 are presented in Table 4.

AIBV was isolated in SPF chicken embryos from rectal swabs positive for *Gammacoronavirus* obtained from a mouflon, a llama, and a spider monkey. Chicken embryos inoculated with rectal samples, as well as those from the five successive serial passages, tested positive for *Gammacoronavirus* by RT-PCR. Sequencing of the products and phylogenetic analysis confirmed the identification of the isolated AIBV (H120 strain). The embryos exhibited characteristic lesions of AIBV infection, including dwarfing, curling, and abnormal down-feather development (Fig. 2).

**Table 3** RT-PCR positive samples for orthocoronaviruses (AIBV, MHV, BCoV, WESV, and HKU10), accession numbers of the detected sequences, genus, species, name, acronym, and strain of the identified viruses

| Animal | Positive samples | | | | Accession number | Isolation | Strain name | Virus name (NCBI Taxonomy ID) | Virus acronym | Species | Genus |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feces | Rectal swab | Oral swab | Nasal swab | | | | | | | |
| Fallow deer | 18.18% (2/11) | 18.18% (2/11) | 0% (0/11) | 0% (0/11) | PQ243315 PQ243316 | No | H120 | Avian infectious bronchitis virus (NCBI:txid11120) | AIBV | *Avian coronavirus* | *Gammacoronavirus* |
| Llama | 50% (1/2) | 50% (1/2) | 0% (0/2) | 0% (0/2) | PQ243317 | Yes | H120 | Avian infectious bronchitis virus (NCBI:txid11120) | AIBV | *Avian coronavirus* | *Gammacoronavirus* |
| Spider monkey | 100% (1/1) | 100% (1/1) | 0% (0/1) | 0% (0/1) | PQ243318 | Yes | H120 | Avian infectious bronchitis virus (NCBI:txid11120) | AIBV | *Avian coronavirus* | *Gammacoronavirus* |
| Mouflon | 6.3% (1/16) | 6.3% (1/16) | 0% (0/16) | 0% (0/16) | PQ243319 | Yes | H120 | Avian infectious bronchitis virus (NCBI:txid11120) | AIBV | *Avian coronavirus* | *Gammacoronavirus* |
| Mouflon | 6.3% (1/16) | 6.3% (1/16) | 0% (0/16) | 0% (0/16) | PQ243321 | No | A59 | Murine hepatitis virus (NCBI:txid11138) | MHV | *Murine coronavirus* | *Betacoronavirus* |
| Dwarf goat | 3.8% (1/26) | 3.8% (1/26) | 0% (0/26) | 0% (0/26) | PQ243320 | No | E-AH65-TC | Bovine coronavirus (NCBI:txid422218) | BCoV | *Betacoronavirus 1* | *Betacoronavirus* |
| Dwarf goat | 3.8% (1/26) | 3.8% (1/26) | 0% (0/26) | 0% (0/26) | PQ243323 | No | Xingguo-74 | Wencheng Sm shrew coronavirus (NCBI:txid1508228) | WESV | Unclassified | *Alphacoronavirus* |
| Pony | 0% (0/1) | 0% (0/1) | 100% (1/1) | 0% (0/1) | PQ243322 | No | 183A | Rousettus bat coronavirus HKU10 (NCBI:txid1241933) | HKU10 | *Bat coronavirus HKU10* | *Alphacoronavirus* |

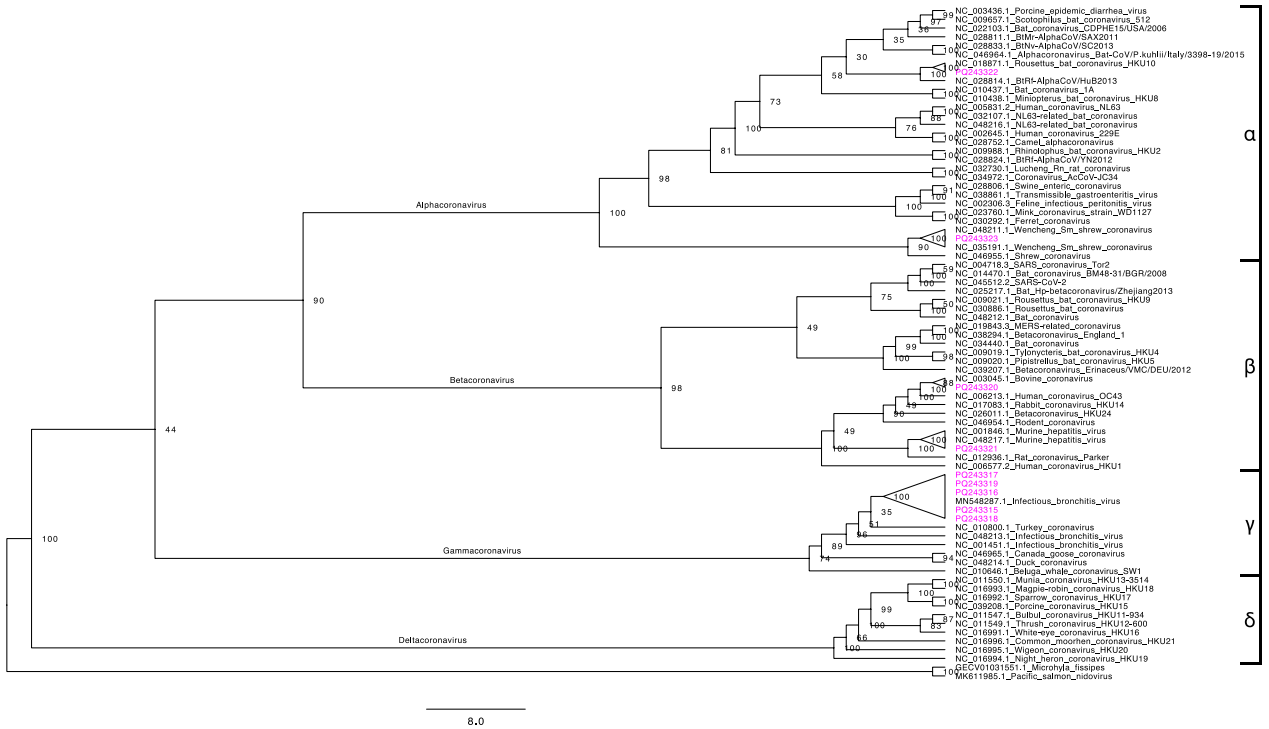


**Fig. 1** Phylogenetic relationships of coronaviruses detected in various animal species in Mexico. The phylogenetic tree was constructed in IQ-TREE using maximum likelihood analysis with 1000 bootstrap replicates, based on nucleotide sequences of the *nsp12* gene. Sequences from *Alphaletovirus microhylae* and *Alphapironavirus salmonis*, representing the subfamilies *Letovirinae* and *Pitovirinae* within the *Coronaviridae* family, were included as outgroups. Accession numbers for sequences generated in this study are highlighted in magenta. The square brackets and Greek letters α, β, γ, and δ identify and delimit the viral sequences belonging to each of the genera within the *Orthocoronavirinae* subfamily

The possibility of environmental or cross-contamination of the positive samples was excluded based on the following considerations: (1) All steps of sampling, transport, storage, and sample processing were performed by personnel fully trained in microbiological sample collection. (2) For species that are difficult to handle, samples were obtained during routine sedation procedures performed for preventive animal care in the included zoos and conservation areas. (3) Samples were collected at different times and locations, and positive animals did not share enclosures. (4) Samples were collected using sterile materials, with swabs taken directly from the animals' body cavities. (5) Positive and negative controls were fully consistent across all RT-PCR assays. (6) The sequences obtained from the samples differed from those of the controls used in the RT-PCR assays. (7) No positive samples were detected in species typically considered as common hosts of the respective coronaviruses (e.g., no avian infectious bronchitis virus was detected in bird samples). (8) RT-PCR detection results from fecal samples and rectal/cloacal swabs were fully consistent. (9) None of the fecal samples or rectal swabs that tested positive by RT-PCR were from animals whose oral and nasal swabs also tested positive.

## Discussion

Coronaviruses can infect a wide range of host species, which are classified based on their epidemiological role in the persistence and transmission of the virus within their ecological niche. Target hosts are the species of primary interest in a specific epidemiological context, where disease control measures are primarily focused. Reservoir hosts are species in which the virus can be maintained indefinitely and that significantly contribute to its transmission to target hosts. Bridge hosts are species that share their habitat with both target and reservoir hosts, facilitating viral transmission between them. Incidental hosts are species that do not play a necessary role in maintaining the virus within the ecosystem nor contribute to its transmission to other hosts [28–30]. Reservoir hosts typically do not exhibit clinical disease, whereas bridge and incidental hosts may develop signs of infection. In contrast, target hosts are more likely to present clinical disease [31]. The study of viruses in uncommon hosts, such as bridge hosts or incidental hosts, can contribute to advances in virology by enhancing our

**Table 4** Identity percentages between the detected *RdRp* nucleotide sequences and those of AIBV, MHV, BCoV, WESV, and HKU10. Sequences pairs with high nucleotide identity are highlighted in bold

| Detected *RdRp* nucleotide sequence identity | Fallow deer (PQ243315) | Fallow deer (PQ243316) | Llama (PQ243317) | Spider monkey (PQ243318) | Mouflon (PQ243319) | Mouflon (PQ243321) | Dwarf goat (PQ243320) | Dwarf goat (PQ243323) | Pony (PQ243322) | AIBV H120 (MN548287.1) | MHV A59 (NC_048217.1) | BCoV E-AH65-TC (EF424616.1) | WESV Xingguo-74 (NC_048211.1) | HKU10 183A (NC_018871.1) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fallow deer (PQ243315) | **100%** | **99.71%** | **99.71%** | **97.98%** | **99.42%** | 21.8% | 25.03% | 30.94% | 36.5% | **100%** | 14.14% | 26.61% | 34.28% | 36.02% |
| Fallow deer (PQ243316) | **99.71%** | **100%** | **99.71%** | **98.27%** | **99.71%** | 21.04% | 24.6% | 30.74% | 36.86% | **99.71%** | 14.37% | 27.07% | 35.42% | 35.45% |
| Llama (PQ243317) | **99.71%** | **99.71%** | **100%** | **97.98%** | **99.42%** | 21.42% | 24.76% | 31.14% | 36.86% | **99.71%** | 13.91% | 26.32% | 35.88% | 34.58% |
| Spider monkey (PQ243318) | **97.98%** | **98.27%** | **97.98%** | **100%** | **97.98%** | 21.25% | 24.33% | 32.53% | 36.86% | **97.98%** | 14.48% | 26.78% | 37.56% | 36.31% |
| Mouflon (PQ243319) | **99.42%** | **99.71%** | **99.42%** | **97.98%** | **100%** | 21.17% | 24.73% | 31.14% | 36.56% | **99.42%** | 14.37% | 27.22% | 35.42% | 35.16% |
| Mouflon (PQ243321) | 21.8% | 21.04% | 21.42% | 21.25% | 21.17% | **100%** | 36.92% | 62.85% | 19.97% | 24.26% | **98.98%** | 35.96% | 40.1% | 17.6% |
| Dwarf goat (PQ243320) | 25.03% | 24.6% | 24.76% | 24.33% | 24.73% | 36.92% | **100%** | 33.43% | 67.55% | 26.61% | 37.85% | **99.73%** | 32.05% | 23.96% |
| Dwarf goat (PQ243323) | 30.94% | 30.74% | 31.14% | 32.53% | 31.14% | 62.85% | 33.43% | **100%** | 42.24% | 35.96% | 63.05% | 33.43% | **98.99%** | 25.96% |
| Pony (PQ243322) | 36.5% | 36.86% | 36.86% | 36.86% | 36.56% | 19.97% | 67.55% | 42.24% | **100%** | 35.45% | 20.27% | 67.17% | 42.57% | **98.49%** |
| AIBV H120 (MN548287.1) | **100%** | **99.71%** | **99.71%** | **97.98%** | **99.42%** | 24.26% | 26.61% | 35.96% | 35.45% | **100%** | 14.14% | 26.61% | 34.28% | 36.02% |
| MHV A59 (NC_048217.1) | 14.14% | 14.37% | 13.91% | 14.48% | 14.37% | **98.98%** | 37.85% | 63.05% | 20.27% | 14.14% | **100%** | 36.81% | 40.23% | 18.24% |
| BCoV E-AH65-TC (EF424616.1) | 26.61% | 27.07% | 26.32% | 26.78% | 27.22% | 35.96% | **99.73%** | 33.43% | 67.17% | 26.61% | 36.81% | **100%** | 31.79% | 23.82% |
| WESV Xingguo-74 (NC_048211.1) | 34.28% | 35.42% | 35.88% | 37.56% | 35.42% | 40.1% | 32.05% | **98.99%** | 42.57% | 34.28% | 40.23% | 31.79% | **100%** | 26.16% |
| HKU10 183A (NC_018871.1) | 36.02% | 35.45% | 34.58% | 36.31% | 35.16% | 17.6% | 23.96% | 25.96% | **98.49%** | 36.02% | 18.24% | 23.82% | 26.16% | **100%** |

**Fig. 2** Characteristic lesions of AIBV infection in chicken embryos inoculated with rectal swab isolates. The embryo on the right shows representative lesions of IBV infection, including dwarfism, curling, and abnormal down-feather development. The embryo on the left is an uninoculated control of the same age

understanding of the global virosphere, elucidating how viruses interact with different host types, and identifying their potential transmission risks among wild and urban host species [32–35].

Coronaviruses can circulate in a variety of wildlife hosts, including bats, rodents, pangolins, and birds, without causing apparent disease. This facilitates their persistence and potential transmission to other species, which may have important implications for the epidemiological surveillance of coronaviruses capable of crossing species barriers [36–46].

Consistent with this perspective, in this study, RNA of several coronaviruses was detected in various wild animals under human care in Mexico that were clinically healthy at sampling. Avian infectious bronchitis virus (H120) was detected in samples from mouflon, fallow deer, llama, and spider monkey; murine hepatitis virus (A59) in samples from mouflon; bovine coronavirus (E-AH65-TC) in samples from dwarf goat; Wénchéng shrew coronavirus (Xingguo-74) in samples from dwarf goat; and Rousettus bat coronavirus HKU10 (183A) in samples from pony. To the best of our knowledge, this is the first report of the presence of these coronaviruses in the mentioned species, highlighting their importance as potentially unrecognized reservoirs for these viruses.

Host shifts, in which a virus jumps from its original host to a new species, are common events in viral evolution. These shifts may necessitate specific adaptations for successful infection in the new host [47]. Some viruses can broaden their host range beyond their common hosts, posing challenges for the implementation of effective disease control measures [48]. Characterizing viral diversity in wildlife and identifying the factors that drive successful cross-species transmission (spillover) are essential objectives for surveillance programs [49].

Coronavirus transmission between species is influenced by a combination of viral, host, and environmental factors, like receptor binding, recombination and mutation, spike protein modifications, natural and anthropic habitat modification, host density and interactions, natural reservoirs and spillover events [50–58]. Some species within the *Deltacoronavirus* genus can infect both birds and mammals. Their ability to spread between such diverse hosts, including pigs and wild birds, underscores their epidemiological significance in the study of coronavirus-host interactions. [59–61].

The ability of coronaviruses to infect new host species is largely determined by their capacity to bind to host cell receptors. For instance, SARS-CoV and SARS-CoV-2 bind to the ACE2 receptor, while MERS-CoV uses DDP4, facilitating their transmission across species [62]. Similarly, porcine deltacoronavirus uses aminopeptidase N (APN) as an entry receptor, which is conserved across multiple species, enabling infection in a broad range

of hosts cells, including human and chicken cells [51]. However, the receptors used by most animal coronaviruses remain unknown. The complexity of interactions between coronavirus spike proteins and their receptors, combined with the viruses' ability to mutate and recombine, poses significant challenges for identifying specific receptors and fully understanding cross-species transmission [63, 64].

The evolutionary history of coronaviruses is characterized by multiple species-jumping events, such as the coronaviruses originating in animals that currently cause human colds [4]. Wild birds have been reservoirs for emerging viruses, such as influenza, West Nile virus, and some *Gamma-* and *Deltacoronavirus* [36, 65]. Their ability to fly reduces the risk of birds becoming prey and increases their ability to colonize new habitats [65]. However, this increased mobility also means that birds can carry pathogens such as viruses thousands of kilometers during their migration, as around 20% of birds are migratory. Birds adapt effectively to urban environments, increasing their population density and favoring pathogens' transmission to humans and other animals [65]. The number of avian species in which coronaviruses have been detected in recent years is considerable. Since the emergence of SARS-CoV in 2002, there has been an increased interest in understanding coronaviruses affecting different species, including birds [4].

Avian infectious bronchitis virus was first identified in the United States in 1930. It usually causes respiratory disease, although some strains can replicate in other epithelia, causing renal and reproductive disorders [66, 67]. AIBV infects various domestic and wild birds such as chicken, pheasant, turkey, brown teal, goose, pigeon, peacocks, parakeets, waterfowl, duck and parrot, quail, guinea fowl, and penguin [68–75]. The detection of AIBV in both domestic and wild birds, even without disease, suggests that the latter may serve as a reservoir and contribute to the spread of AIBV on a global scale [4].

Previously reported serotypes of AIBV in Mexico include MX/BL56-19/UNAM/96 (MX/5697/99), MX/UNAM97/97, MX/07484/98, and MX/7277/99 [71, 76, 77]. The H120 strain of AIBV identified in this study from samples collected from naturally infected mouflon, fallow deer, llama, and spider monkey was originally reported in the Netherlands in 1960 and is currently circulating in China. The H120 strain was derived from an AIBV of the Massachusetts serotype, attenuated through 120 passages in chicken embryos [78].

The ability of BR-I and Mass strains of AIBV, including the H120 strain, to experimentally infect BALB/c and A/J mice has previously been investigated. Viral RNA was detected at 14 days post-infection in the lungs, trachea, nasal sinuses, and duodenum of intranasally inoculated mice. While the infection did not produce clinical signs or lesions, histopathological changes such as interstitial pneumonia, edema, and perialveolar inflammatory infiltrate were observed [79]. Additionally, AIBV has been replicated in cell lines derived from other species, including cat (CRFK), African green monkey (VERO), and human (HeLa) [80–82].

Bovine coronavirus (BCoV) was originally identified, isolated, and attenuated in FBK cells in 1973 from the feces of cattle with diarrhea [83]. BCoV is associated with neonatal diarrhea, winter dysentery, and shipping fever, but it can also be found in the respiratory and digestive tracts of healthy cattle [84–86]. Although BCoV and bovine-like CoVs cannot be distinctly differentiated through comparative genetic studies due to their high genetic similarity, genotyping enables the identification of various sublineages based on the year and place of isolation, regardless of the type of disease they produce. This suggests that clinical presentation may result from complex interactions among pathogens, hosts, and environmental factors [87–91].

BCoV and bovine-like CoVs have been identified in various species, including alpaca, dromedary camel, wapiti, giraffe, Himalayan tahr, llama, musk oxen, nyala, sable antelope, sambar deer, sheep, goat, sika deer, sitatunga, water buck, water buffalo, water deer, white-tailed deer, wisent, yak, dogs, and humans [84, 92, 93]. Experimental infection of one-day-old SPF chickens and turkeys with the BCoV strain DB2 has also been described, causing enteric infection signs and lesions 48–72 h post-inoculation, generating antibodies, and conferring protection against infection upon re-inoculation [94]. The E-AH65-TC strain of BCoV identified in this study from samples collected from a naturally infected dwarf goat was originally described as an enteric BCoV isolate from feedlot calves with enteric disease in Ohio [95].

Rousettus bat coronavirus HKU10 was first identified in 2012 in the fruit bat *Rousettus leschenaulti* in Guangdong, China, and has since been identified in multiple bat species, highlighting its potential for cross-species transmission. Initially, this virus was detected in two bat species from different suborders: the frugivorous *Rousettus leschenaultii* and the insectivorous *Hipposideros pomona*, representing a notable case of transmission between phylogenetically distinct hosts [96]. The geographic distribution and evolutionary history of HKU10 remain incompletely understood. However, recent studies have expanded knowledge of its presence in several provinces of China and in Laos. The virus has also been detected in additional bat species, such as *Aselliscus stoliczkanus* and *Hipposideros larvatus*, suggesting greater genetic diversity than previously recognized. Notably, six novel viral lineages have been identified, particularly in Yunnan

Province, indicating that this region may serve as a natural reservoir for the virus [97]. The cross-species transmission of HKU10 is of significant interest, as it provides insight into how coronaviruses adapt to new hosts [96].

Wénchéng shrew coronavirus was first described in 2017 in the Asian house shrew *Suncus murinus* in Jiangxi and Zhejiang, China. Phylogenetic analysis revealed that WESV is a highly divergent member of the *Alphacoronavirus* genus, with its spike (S) gene forming a genetically distinct cluster compared to known coronaviruses. Additionally, the WESV genome contains a unique NS7 gene that exhibits no sequence similarity to genes of other known viruses, suggesting that shrews could serve as natural reservoirs of coronaviruses and may have played a significant role in the evolution of these viruses [98]. Currently, there are no reports of diseases caused by the infection with HKU10 or WESV.

Murine hepatitis virus was first identified in China. It causes hepatic and neurological signs in rodents. [99]. MHV has been used as a model to study the mechanisms of interspecies transmission of coronaviruses. Studies have demonstrated that episodic evolution and positive natural selection are critical for the interspecies transfer of MHV. It has been observed that viral variants, such as MHV-H2, can efficiently replicate in cells from commonly non-permissive species, including Syrian hamster kidney cells and Chinese hamster ovary cells, among others [100, 101]. Although it spreads efficiently among rodents, there are no reports of its natural transmission from rodents to other species [99].

To date, AIBV has not been detected in any mammalian species. This study is the first to report natural infection of ruminants and wild primates under human care with AIBV, as well as their capacity to shed active virus. The ability to predict the susceptibility to coronaviruses of various cell types, organs, and systems across species, as well as their routes of viral shedding, remains highly limited. Enhancing the detection of these widely diverse viruses, along with their common and uncommon hosts, both known and yet to be discovered, requires continued investigation of natural coronavirus infections in domestic and wild animals, as well as in humans. Such research is essential for advancing our understanding of the complex virus-host-environment dynamics that ultimately impact One Health.

The primary limitation of this study is the need for additional observational and experimental research, as the current scope restricts the depth of analysis and the conclusions that can be drawn from the presented data. Further studies are required to determine the relevance of the detected coronaviruses in these uncommon hosts within their respective ecological niches, as well as each host's potential role in viral transmission and disease spread to other species, which may have broader implications for wildlife conservation and public health.

#### Author contributions

Conceptualization: JMG, JAPT, FJBA; Data curation: JMG; Formal Analysis: JMG; Funding acquisition: FJBA; Investigation: JMG; Methodology: JMG, JAPT; Project administration: JMG, FJBA; Resources: FJBA, YLV; Supervision: FJBA, YLV; Validation: JMG; Visualization: JMG, JAPT; Writing—original draft: JMG; Writing—review & editing: JMG, JAPT, LVS, FJBA, YLV.

#### Funding

Funding for this study was provided by Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica IN202522 "Detección y caracterización genética de coronavirus en animales en México" from the Universidad Nacional Autónoma de México (UNAM). This work was supported by a doctoral scholarship for Jocelyn Medina-Gudiño from the Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT).

#### Availability of data and material

Sequence data that support the findings of this study have been deposited in the National Institutes of Health genetic sequence database with the primary accession numbers PQ243315-PQ243323.

#### Code availability

Not applicable.

### Declarations

#### Ethics approval and consent to participate

The Internal Committee for Animal Care and Use (CICUA) approved the sample collection procedures under protocol number SICUAE.DC-2023/4–1. Facultad de Medicina Veterinaria y Zootecnia. Universidad Nacional Autónoma de México (FMVZ-UNAM).

#### Competing interests

The authors declare no competing interests.

Received: 13 November 2024 Accepted: 3 April 2025
Published online: 26 April 2025

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