Divergent Genotype of Hepatitis A Virus in Alpacas, Bolivia, 2019

Talitha Veith, L. Fabian Beltran-Saavedra, Tobias Bleicker, Marie Luisa Schmidt, José L. Mollericona, Kim Grützmacher, Rob Wallace, Jan Felix Drexler, Christian Walzer, Terry C. Jones, Christian Drosten, Victor Max Corman

Hepatitis A virus (HAV) is a common human pathogen found exclusively in primates. In a molecular and serologic study of 64 alpacas in Bolivia, we detected RNA of distinct HAV in ≈9% of animals and HAV antibodies in ≈64%. Complete-genome analysis suggests a long association of HAV with alpacas.

Hepatitis A virus (HAV) causes acute hepatitis in humans worldwide; ≈159 million infections and ≈39,000 deaths were associated with HAV in 2019 (*1*), despite an available and effective vaccine (*2*). HAV infection and vaccination normally induce lifelong immunity (*2*).

The genus *Hepatovirus* (family *Picornaviridae*) consists of 9 species, designated A–I (*3*). Species B–I have been detected in small wild mammals, in seals, and in a domestic goat (*4*–*8*). HAV strains of *Hepatovirus A* have been exclusively associated with primates. All human hepatoviruses pertain to species A. Within species A, genotypes I–III are found in humans, and genotypes IV–VI are found in monkeys (*9*). No wildlife or livestock reservoir has been described for human HAV.

We screened 64 alpacas and 6 llamas in Bolivia for viruses and detected a divergent nonprimate genotype of *Hepatovirus* species A. We provide serologic evidence for a high frequency of HAV infection in New World camelids.

The Study

We collected serum and feces samples from 64 alpacas and 6 llamas in Bolivia within the Apolobamba national protected area near the Bolivia-Peru border in 2019 (Figure 1). We tested RNA from 70 serum samples and 69 fecal samples stored in RNAlater (ThermoFisher Scientific, https://www.thermofisher.com)

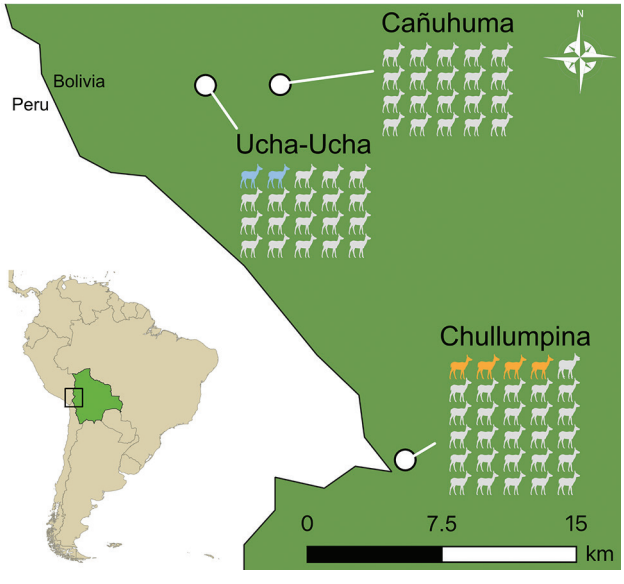

Figure 1. Alpaca and llama sample collection sites in study of hepatitis A virus in alpacas and llamas, Bolivia, 2019. The number of animals sampled per site is shown, 70 animals in total. Colored icons indicate alpaca HAV-positive animals by quantitative reverse transcription PCR. Inset shows locations of Bolivia and study site in South America.

Author affiliations: Charité-Universitätsmedizin Berlin—corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany (T. Veith, T. Bleicker, M.L. Schmidt, J.F. Drexler, T.C. Jones, C. Drosten, V.M. Corman); Wildlife Conservation Society, La Paz, Bolivia (L.F. Beltran-Saavedra, J.L. Mollericona, R. Wallace); Wildlife Conservation Society, New York, New York, USA (L.F. Beltran-Saavedra, J.L. Mollericona, R. Wallace, C. Walzer); Museum für Naturkunde Berlin/Leibniz-Institute for Evolution and Biodiversity Science, Berlin (K. Grützmacher); Deutsche Gesellschaft für Internationale Zusammenarbeit GmbH, Bonn, Germany (K. Grützmacher); German Centre for Infection Research (DZIF), Berlin (J.F. Drexler, C. Drosten, V.M. Corman); University of Veterinary Medicine Vienna, Vienna, Austria (C. Walzer); University of Cambridge, Cambridge, UK (T.C. Jones); Labor Berlin Charité–Vivantes GmbH, Berlin (V.M. Corman)

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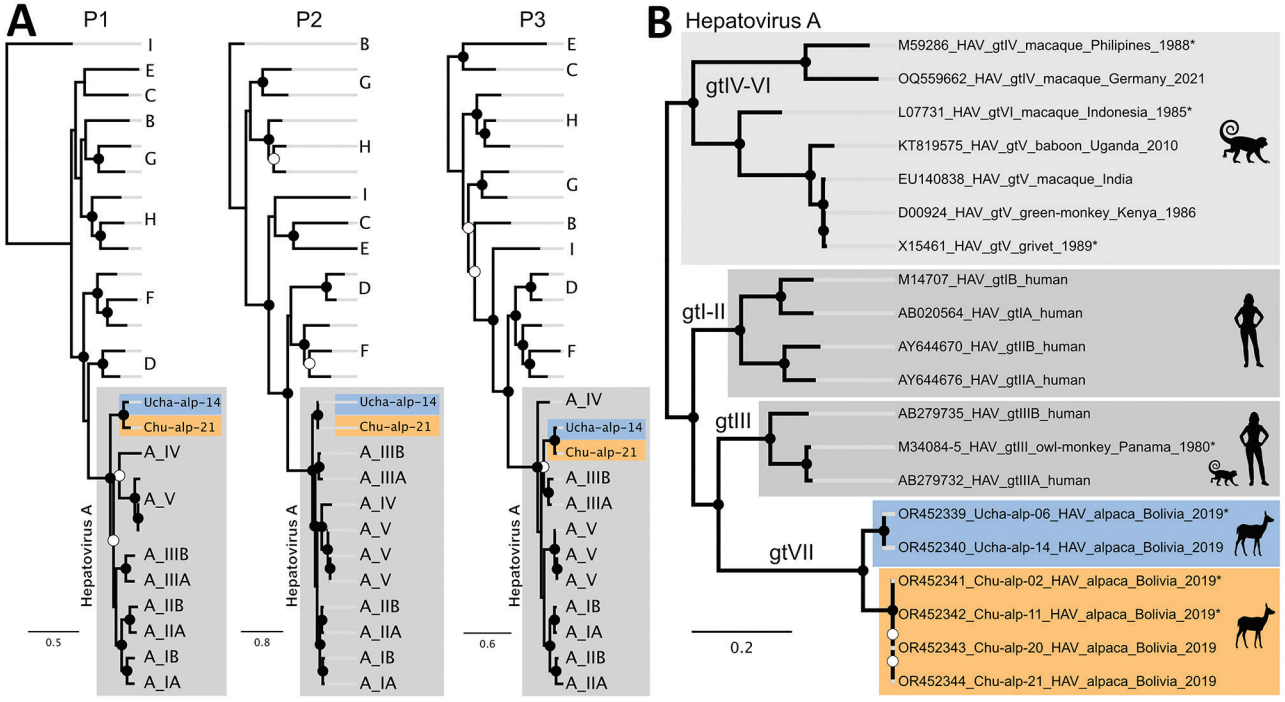

Figure 2. Phylogenetic analyses of alpaca HAV from Bolivia, 2019, in the context of other HAV sequences. Alpaca HAV sequences are colored by site, Ucha-Ucha in blue, Chullumpina in orange. A) Maximum-likelihood phylogenies of alpaca HAV in the context of other hepatovirus species. P1, P2, and P3 (picornavirus regions typically separated by recombination breakpoints [*10*]) nucleotide alignments were made using Clustal Omega 1.2 (http://www.clustal.org/omega) in Geneious Prime 2023.1.2 (https://www.geneious.com). ModelFinder, incorporated in IQ-TREE 1.6.12 (http://www.iqtree.org), was used to determine the best-fitting nucleotide substitution model (general time reversible model with a discrete gamma model with 4 rate categories, invariable sites, and empirical base frequencies) according to the Bayesian Information Criterion. IQ-TREE 1.6.12 was used to calculate the phylogenetic tree. The trees were rooted with *Hepatovirus* species I (P1), B (P2), and E and C (P3) respectively. Solid black circles denote ultrafast bootstrap support of ≥90 for the preceding branch; open circles denotes ultrafast bootstrap support between 70 and 90. GenBank accession numbers by genogroup: I, KT452658; E, KT452735; C, KT452742; B, KR703607; G, KT452730 and KT452729; H, KT452691, KT452714, and KT877158; F, KT229611, MG181943, and KT452685; D, KT452644 and KT452637; A, OQ559662, KT819575, EU140838, D00924, AB279735, AB279732, AY644670, AY644676, M14707, and AB020564. B) Maximum-likelihood phylogeny of alpaca HAV in the context of other *Hepatovirus A* genotypes. Complete genomes and partial genomes (asterisks) were used. The tree was calculated as in panel A, by using a transition model with AC=AT and CG=GT, with a discrete gamma model with 4 rate categories, invariable sites, and empirical base frequencies and rooting with *Hepatovirus* species A genotypes IV–VI. Chu-alp-11, included in this tree, tested negative in quantitative reverse transcription PCR but positive in high-throughput sequencing and pan-*Hepatovirus* PCR (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-1123-App1.pdf). HAV, hepatitis A virus.

in pools of 8–10 using Illumina (https://www.illumina.com) high-throughput sequencing. In 3 of 16 pools, we detected matches with HAV and investigated this finding in detail.

We tested all individual samples for alpaca HAV RNA with a novel specific quantitative reverse transcription PCR (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-1123-App1.pdf). Testing did not

Table 1. Nucleotide distance matrix of complete HAV genomes in alpacas, Bolivia, 2019*

Genome	Ucha-alp-14	Chu-alp-21	IA	IB	IIA	IIB	IIIA	IIIB	V
Chu-alp-21	**7.6**								
IA	18.6	19							
IB	18.6	19.1	**8.7**						
IIA	19.5	20.1	14.2	14.2					
IIB	18.7	19.2	14.1	14.2	**9.2**				
IIIA	18.2	18.6	17.3	17.3	17.3	17.2			
IIIB	18.1	18.8	17	17	17.4	17.3	**10.8**		
V	20.8	21	19.1	19	19.5	19	19.5	19.3	
VI	21.4	22	20.5	20.8	21	21	20.3	19.9	20.3

*Alpaca HAV and existing *Hepatovirus A* genotypes were included where complete genomes were available. Bold indicates intra-genotype distances. Genomes were aligned using ClustalOmega 1.2 (http://www.clustal.org/omega) in Geneious Prime 2023.1.2 (https://www.geneious.com). Nucleotide distances, given in percentages, were calculated in Geneious Prime 2023.1.2. Accession numbers used: IA, AB020564; IB, M14707; IIA, AY644670; IIB, AY644676; IIIA, AB279732; IIIB, AB279735; V, EU140838; VI, OQ559662. HAV, hepatitis A virus.

Table 2. Results of hepatis A virus IgG ELISA in serum of alpacas and llamas, Bolivia, 2019

Animal	No.	Reactive serum samples/total tested (%)		
		Age <2 y	Age >2 y	Total
Llamas	6	0	4/6 (66.7%)	4/6 (66.7%)
Alpacas	64	12*/26 (46.2%)	29/38 (76.3%)	41*/64 (64.1%)
Total	70	12*/26 (46.2%)	33/44 (75.0%)	45*/70 (64.3%)

*One alpaca serum sample from an alpaca <2 years of age (Chu-alp-06, positive in quantitative reverse transcription PCR) had an unclear ELISA result in 2 tests and a negative result in a 3rd test.

detect alpaca HAV in any of the 6 llamas. In contrast, alpaca HAV RNA was detected in 6/64 (9.4%) alpacas: in 5 of 64 (7.8%) serum samples and 5 of 63 (7.9%) feces samples. In 4 of those 6 alpacas, serum and feces samples were both positive. Concentrations of alpaca HAV RNA were up to 3.2×10^5 RNA copies/mL in serum and 3.6×10^5 RNA copies/mL in feces (Appendix Figure 1).

We further processed all alpaca HAV–positive samples for complete genome sequencing using undirected Illumina NextSeq sequencing (https://www.illumina.com), HAV-specific in-solution sequence capturing, and GridION reverse transcription PCR amplicon sequencing (Oxford Nanopore Technologies, https://nanoporetech.com) (Appendix Table). We generated 2 complete and 4 partial alpaca HAV genomes for further analyses (GenBank accession nos. OR452339–44). In a phylogenetic tree, alpaca HAVs form a distinct monophyletic clade within other *Hepatovirus A* sequences (Figure 2, panel A). Implied membership in the species *Hepatovirus A* is confirmed by sequence comparison. The alpaca HAV polyprotein amino acid sequence is 9%–11% distant from other *Hepatovirus A*, well within the species demarcation criterion of 30% set by the International Committee on Taxonomy of Viruses (*3*). In addition, alpaca HAV's distance from established HAV genotypes (18%–22%) is similar to HAV genotypes' distances from each other (14%–21%) (Table 1). Alpaca HAV regions are also similarly distant to all 3 human HAV genotypes across the genome; highest divergence is in the N terminus of 2C and in 3A (Appendix Figure 3). Therefore, alpaca HAV likely represents a distinct genotype, tentatively named genotype VII (gtVII), within *Hepatovirus A*.

We detected alpaca HAV at 2 of 3 locations: Ucha-Ucha and Chullumpina, which are ≈20 km apart (Figure 1). The separation is reflected in phylogenetic analysis, in which 2 monophyletic clades of alpaca HAV sequences correspond to these geographic sites (Figure 2, panel B). Representative complete genome sequences from each site (Ucha-alp-14 and Chu-alp-21) are 7.6% distant in nucleotide sequence, similar to the distance between subgenotypes A and B of human genotypes I–III (≈9%–11%). Thus, alpaca HAVs from both sites could be classified into 2 subgenotypes, gtVIIA and gtVIIB (Table 1). Between both alpaca HAVs, 94.8% of nucleotide changes in the coding region are synonymous, and the dN/dS ratio, calculated with the Python dnds module (https://pypi.org/project/dnds), is 0.009, suggesting purifying selection.

Currently described HAV genotypes commonly belong to a single serotype (*11*,*12*). Neutralization epitopes are located in the capsid proteins VP1–3 (*13*), and most amino acids of those epitopes are conserved between HAV genotypes I–V and alpaca HAV (Appendix Figure 2). Thus, we were able to conduct a serologic analysis of alpaca and llama serum samples using an HAV IgG ELISA employing human HAV antigens (Mediagnost, https://mediagnost.de). Of 64 alpaca serum samples, 41 (64.1%) were reactive for HAV IgG, including the serum of 13 (81.3%) of 16 alpacas from Cañuhuma, where no alpaca tested positive for alpaca HAV RNA (Table 2; Appendix Figure 4). Of the 6 llama serum samples, 4 (66.7%) were reactive for HAV IgG (Table 2; Appendix Figure 4). In line with lifetime buildup of immunity, the proportion of seroreactive alpacas increased with age. Alpacas >2 years of age were more likely to have HAV antibodies than were younger alpacas; antibodies were present in 76.3% of older alpacas, compared with 46.2% of younger alpacas ($\chi 2 = 6.8$, d.f. = 2, n = 64; p = 0.034).

No sampled animal showed obvious clinical signs of a systemic or hepatic infection. However, we were not able to collect more data on the pathogenicity of HAV infection in alpacas. Other limitations of our study are the small number of samples and the limited geographic sampling range.

Conclusions

We describe a nonprimate host association of a divergent HAV genotype in alpacas. We detected alpaca HAV RNA in both serum and feces samples, as is typically seen in acute human HAV infections. Signs of seroconversion were common, and seroreactivity increased with age. The relatively high seropositivity rate suggests that infection with alpaca HAV is common. Sequences of alpaca HAV are diversified at the nucleotide level but conserved at the amino acid level. The nucleotide diversity is consistent with a long evolutionary association of HAV with alpacas.

Hepatoviruses have been observed to undergo host-switching (*14*). A spillover event might also have been involved in HAV emergence in alpacas. However,

our data are inconclusive regarding the origin of alpaca HAV and whether alpaca HAV spilled over to or from humans. More camelid and nonhuman primate HAV sequences are needed to resolve this question.

Detecting antibodies using a HAV ELISA kit with human HAV antigens suggests that alpaca HAV might belong to the same serotype as genotypes I–VI. HAV vaccination might thus provide protection from a potential alpaca HAV spillover from alpacas into humans and vice versa. Bolivia is currently considered an area of high-intermediate endemicity of HAV (*15*); increased local outbreaks and a higher burden of HAV-associated disease are expected. With that in mind, HAV vaccinations, especially for camelid handlers, should be considered to reduce spillover risk.

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About the Author

Ms. Veith is a virologist at the Institute of Virology, Charité–Universitätsmedizin Berlin, Berlin, Germany. Her research interests include disease ecology and virus diversity studies.

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Address for correspondence: Victor Max Corman, Campus Charité Mitte, Charitéplatz 1, 10117 Berlin, Germany; email: victor.corman@charite.de

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Appendix

Material and methods

Sampling

Sample collection from domestic new world camelids (alpacas and llamas) was conducted during routine scheduled health checks after consultation with the breeders who owned the herds, on April 16 and 17, 2019. For sample collection, animals were briefly physically restrained using established traditional methods for health checks, sampled, and then released. Fecal samples were taken from the rectum and directly transferred into cryotubes containing RNAlater (Thermo Fisher Scientific, Waltham, USA). Blood was collected from the right jugular vein, centrifuged, and serum was transferred into cryotubes. Samples were immediately cooled for 1 month and then stored at −80°C.

Nucleic acid isolation

Nucleic acid (NA) was isolated either using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) or the MagNA Pure DNA and Viral NA Small volume kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. All NA isolations of pools and individual samples used for quantitative reverse transcription PCR (RT-qPCR) were carried out using the MagNAPure system, with 100 µL input volume for serum and fecal samples, and elution in 100 µL. Repeat individual isolations were performed on QIAamp Viral RNA column, with 140 µL input volume for serum, 70 µL input volume for fecal samples, and 70 µL elution volume for serum and feces.

RT-qPCR and in-vitro transcript

Isolated nucleic acids were tested for alpaca HAV presence using specific RT-qPCR primers and probe (Appendix Table). For quantification, an alpaca HAV-specific in-vitro transcript (IVT) was designed (Appendix Table). Double stranded DNA comprising the PCR target region as well as a T7-promoter sequence was synthesized by Integrated DNA Technologies (Leuven, Belgium). In-vitro transcription was performed using the MEGAScript T7 Transcription kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions, using 100 ng of DNA as input. The 37°C incubation was carried out for 4 hours. The DNA was digested using DNase I-XT (New England Biolabs, Frankfurt am Main, Germany) according to manufacturer's instructions for 30 minutes at 37°C. The RNA was then cleaned up using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, with an elution volume of 50 µL. The eluate was diluted 1:10 in RNase-free water, and its RNA concentration was determined using the Qubit HS RNA kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. The RNA was diluted to 10^9 copies/µL in water containing 10 ng/µL carrier-RNA (Qiagen, Hilden, Germany). A 1:10 dilution series in carrier-RNA containing RNase-free water was prepared. Through alpaca HAV RT-qPCR (described in detail below) of the dilution series on LightCycler 480 (Thermo Fisher Scientific, Waltham, USA), a calibration curve was created using the fit point method. The RT-qPCR was able to detect specific RNA down to 1 RNA copy/µL (equivalent to 5 copies/PCR reaction). To exclude leftover presence of DNA, a qPCR without reverse transcription was performed. IVT concentrations of 10^7 RNA copies/µL and below did not contain detectable aHAV DNA.

Isolated serum and fecal samples were tested for alpaca HAV presence and RNA concentration by RT-qPCR using SuperScript™ III One-Step (Thermo Fisher Scientific, Waltham, USA). Five µL RNA was added to a mix containing 2.6 µL of RNase-free water, 12.5 µL of 2x reaction mix, 1 µL of BSA (1 mg/mL), 0.4 µL of MgSO4 (50 mM), 1 µL of each primer (10 uM), 0.5 µL of the probe (10 uM), and 1 µL of SuperScriptIII/P.Taq enzyme. Reverse transcription was performed at 55°C for 10 minutes, followed by 3 minutes of 95°C. 45 cycles of 95°C for 15 seconds and 58°C for 30 seconds were run. Quantification took place at 58°C. alpaca HAV IVT at a concentration of 10^3 copies/µL was tested in every run. Alpaca HAV RNA concentrations were determined through the created external calibration curve.

Illumina HTS and in-solution bait capturing

RNA pools, and RNA isolates in which alpaca HAV was detected, were Illumina (San Diego, USA) shotgun-sequenced. RNA concentrations were measured using the Qubit RNA HS Assay kit (Thermo Fisher Scientific, Waltham, USA). From up to 100 ng per sample, libraries were produced using the KAPA RNA HyperPrep kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. The RNA was fragmented for 6 minutes at 85°C, and the indexed libraries were PCR-amplified for 9–12 cycles. Libraries were pooled at equimolar ratio. Sequencing was performed on Illumina NextSeq (NextSeq 550 or NextSeq 1000/2000) with 150 or 300 cycles (Illumina, San Diego, USA).

To enrich alpaca HAV viral reads, a custom-designed bait-set against hepatitis viruses A-E was used. This set is based on all human hepatitis virus sequences available in the NCBI database, including HAV genotypes I-III. It contains over 77,000 individual baits (sequences available upon request), each 70 nucleotides long, and together they double-tile each input sequence. Bait sequences were collapsed so that they have a maximum nucleotide similarity of 90% for hepatitis C virus, and 97% for other hepatitis viruses. The myBaits Custom Target Capture Kit v5 was ordered from, and partially designed by Arbor Biosciences (ArborSci, Ann Arbor, USA). Capturing was performed following the manufacturer's instructions, with hybridization at 60°C and 18–22 cycles of PCR amplification.

Nanopore Sequencing

SuperScript™ III One-Step (Thermo Fisher Scientific, Waltham, USA) PCRs were carried out according to manufacturer's instructions to yield amplicons for sequencing. Primer sequences can be found in the Appendix Table. Amplicons were cleaned up using KAPA Pure Beads (Roche Diagnostics, Basel, Switzerland) in a 1:1 concentration, according to manufacturer's instructions. Subsequently, libraries were prepared from the amplicons using kit SQK-PSK004 (Oxford Nanopore Technologies Limited, Oxford, UK), and multiplexing was performed using the SQK-PBK004 barcoding kit (Oxford Nanopore Technologies Limited, Oxford, UK) according to manufacturer's instructions. Libraries were sequenced on a GridION (Oxford Nanopore Technologies Limited, Oxford, UK) using the FLO-FLG001 flongle flow cell (Oxford Nanopore Technologies Limited, Oxford, UK).

ELISA

The commercial ELISA kit E10 antiHAV of Mediagnost (Reutlingen, Germany) was used to detect total antibodies against HAV. Samples were tested at a 1:10 dilution in the kit-contained dilution buffer. 100 µL of 1:10 dilution was tested according to manufacturer's instructions. The optical density (OD) was measured at 450 nm (for the reaction) and 620 nm (for background). The background OD was subtracted from the reaction OD. A blank control was included, and its OD450-OD620 was subtracted from all OD450-OD620 values, and the final value is called adjusted OD. Positive and negative controls (PC and NC respectively), as well as standards, were used undiluted. (PC+NC)/2 was used as the cutoff for positive and negative results, as suggested by the manufacturer for human samples. Samples ranging within +/− 10% of the cutoff value (termed the cutoff range) were considered to have an unclear result. All values were within the ranges expected according to the manufacturer. The ELISA used is a competition-ELISA, thus samples with adjusted OD below the cutoff are reactive, while samples with OD above the cutoff are non-reactive.

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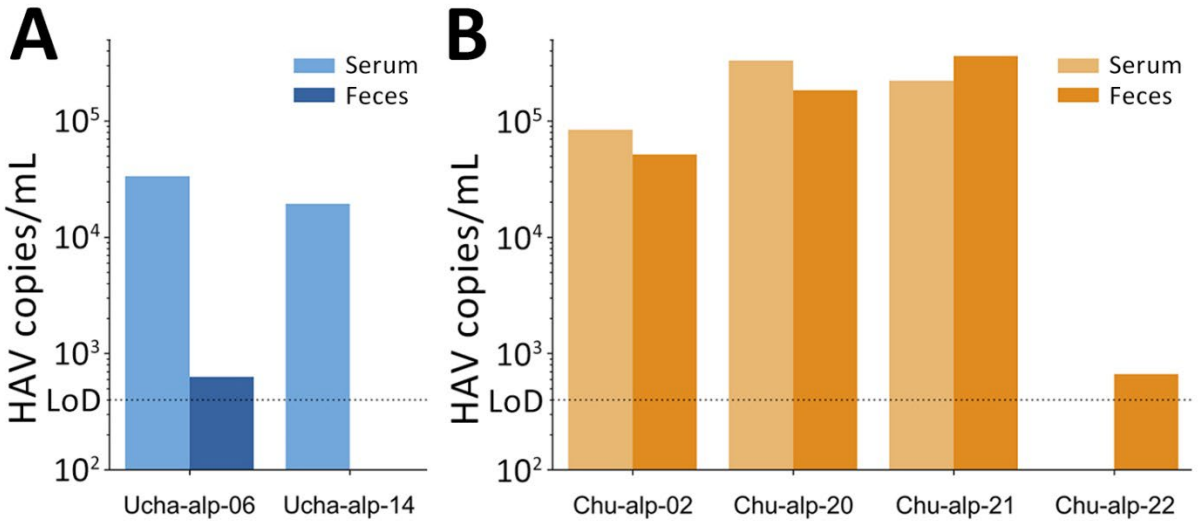
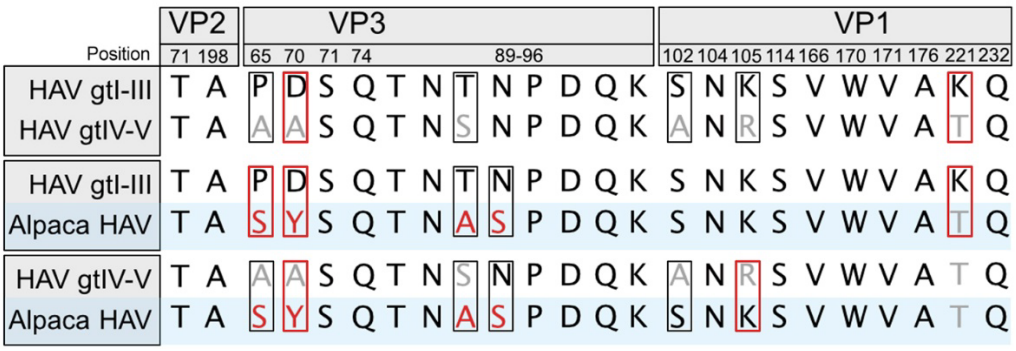
Appendix Table. Oligonucleotide sequences used in this study, including primers, probes, and in-vitro transcript

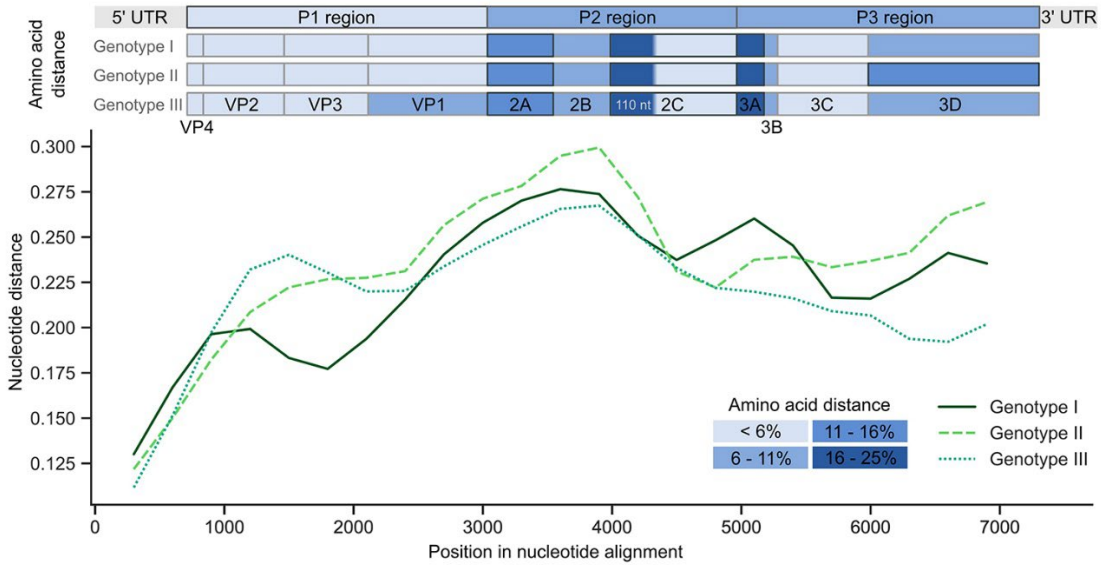
Name	Sequence 5′-3′	Comments
HAV-Alp-RTq-F	ATAGGGTAACAGCGGCGGATA	Primers and probe adapted from human HAV FDA RT-qPCR assay (https://www.fda.gov/media/160119/download)
HAV-Alp-RTq-Probe	CCTTTCAACGCCGGAGGACTGACTCTC	
HAV-Alp-RTq-R	CAATCCACTCAATGCATTCACTGG	
HAV-Alp-01-F	CCGTTTGCCTAGGCTATAGGC	Primers used to generate complete genome of Ucha-alp-14
HAV-Alp-01-R	GCTGTTACCCTATCCAAAGCATC	
HAV-Alp-02-F	CTACCAGATGACAAATGCCAGTCC	
HAV-Alp-02-R	CTGGATGTTGTAATTCCAACCTGAG	
HAV-Alp-03-F	CAGGTTGGAATTACAACATCCAGAG	
HAV-Alp-03-R	CTAGAGCGATTCTGTTCATCACTC	
HAV-Alp-04-F	GTAAATCTTGCTGATAGAATGCTGG	
HAV-Alp-04-R	CAAGTCCACTCCTTTCTGATACTG	
HAV-Alp-05-F	GGATGGATATAGTGGCCAGTTAGTG	
HAV-Alp-05-R	CACTAATAACCAGTCATCCTTGATTCC	
HAV-Alp-06-F	GGAGCTCTTGTATCTTCCAATCAG	
HAV-Alp-06-R	GCTGGTCCCATTGTCTATCAGG	
HAV-Alp-07-F	CCGTCTGGTTCTCCTTGTACG	
HAV-Alp-07-R	CACTGATCATTGAATCTCATTCTCC	
HAV-panHepato-F	GAGATAYCAYACWTATGCIAGATTTGG	Primers from (*1*) were used to generate VP2-VP3 sequence of Ucha-alp-06 and Chu-alp-11
HAV-panHepato-Rnest	CTRAATTCRTTICTCATCATYTGTG	
HAV-panHepato-R	GACATYTTIGCYCTIGCATCYTC	
HAV-Alp-IVT	TAATACGACTCACTATAGGGAGAGTAAAACGACGGCCA GTGAATGGGTGAAACCTCTTAAGCTAATACTTCTATGA AGAGATGCTTTGGATAGGGTAACAGCGGCGGATATTG GTGAGTTGTTTGACAAAAACCTTTCAACGCCGGAGGAC TGACTCTCATCCAGTGAATGCATTGAGTGGATTGTATG TCAGGGCTGTCTCTAGGCTTAATTTCTGACCTCTCTGT GCGTCATAGCTGTTTCCTGTGTG	In-vitro transcript sequence includes a T7 promoter sequence, a M13 forward primer sequence, 185 bp of alpaca HAV sequence including primer and probe binding sites, and a M13 reverse primer sequence



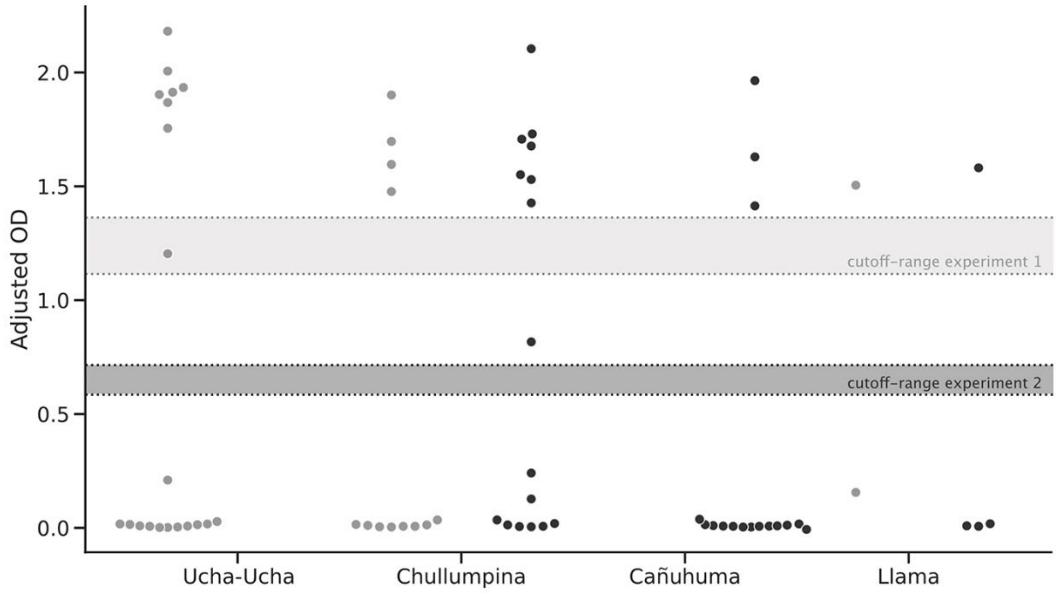
Appendix Figure 1. Alpaca HAV RNA concentration in copies/mL per site, in serum and feces samples. The lower limit of detection (LoD) is marked as a line.



Appendix Figure 2. Comparison of Hepatovirs A genotypes in amino acids that determine the HAV neutralization phenotype (*1*,*2*). Red letters denote epitopes in which alpaca HAV differs from other HAV genotypes, gray where HAV gtIV-V differs from HAV gtI-III or where alpaca HAV has the same amino acid as HAV gtIV-V. The colors of the boxes indicate Sneath's index of amino acid difference of the two amino acids in the box (red >20, black < = 20).



Appendix Figure 3. Alpaca HAV (Chu-alp-21) sequence comparison to human HAV genotypes I-III (AB020564, AY644670, and AB279732, respectively) across the genome on amino acid (blue) and nucleotide basis (green). The polyprotein amino acid sequences were extracted in Geneious Prime 2023.1.2, the genotype I-III sequences were pairwise aligned with alpaca HAV, and the percentage distance in all regions and open reading frames was calculated. The percentage distance describes all nonidentical sites out of the total sites. To analyze the nucleotide distance, all four sequences were multiple aligned using MAFFT 1.5.0 in Geneious Prime 2023.1.2, and patristic distances were calculated using RDP4.101, with chu-alp-21 as reference, a sliding window of 600 nucleotides and 300 nucleotides step size. The distance data was visualized with seaborn 0.12.2 within Python 3.11.4.



Appendix Figure 4. Raw data of HAV IgG ELISA for the 70 individual alpaca and llama serum samples. Adjusted OD represents: (sample OD450 - sample OD620) - (blank control OD450 - blank control OD620). Adjusted ODs above the cutoff-range are nonreactive, ODs below are reactive, and ODs within the range have unclear reactivity. Two separate experiments were carried out to measure all samples, shown in light and dark gray respectively. Thus, the cutoff-ranges differ between the two experiments, shown for each experiment in background color of light or dark gray. Sample Chu-alp-06 was measured in both experiments to ensure repeatability.