

1 **Differential infection patterns and recent evolutionary origins of equine hepaciviruses in**
2 **donkeys**

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69 **Abstract**

70 The hepatitis C virus (HCV) is a major human pathogen. Genetically related viruses in
71 animals suggest a zoonotic origin of HCV. The closest relative of HCV is found in horses
72 (termed equine hepacivirus, EqHV). However, low EqHV genetic diversity implies relatively
73 recent acquisition of EqHV by horses, making a derivation of HCV from EqHV unlikely. To
74 unravel the EqHV evolutionary history within equid sister species, we analyzed 829 donkeys
75 and 53 mules sampled in nine European, Asian, African and American countries by
76 molecular and serologic tools for EqHV infection. Antibodies were found in 278 animals
77 (31.5%), and viral RNA was found in 3 animals (0.3%), all of which were simultaneously
78 seropositive. A low RNA prevalence in spite of high seroprevalence suggests predominance
79 of acute infection, a possible difference from the mostly chronic hepacivirus infection pattern
80 seen in horses and humans. Limitation of transmission due to short courses of infection may
81 explain the existence of entirely seronegative groups of animals. Donkey and horse EqHV
82 strains were paraphyletic and 97.5-98.2% identical in their translated polyprotein sequences,
83 making virus/host co-speciation unlikely. Evolutionary reconstructions supported host
84 switches of EqHV between horses and donkeys without the involvement of adaptive
85 evolution. Global admixture of donkey and horse hepaciviruses was compatible with
86 anthropogenic alterations of EqHV ecology. In summary, our findings do not support EqHV
87 as the origin of the significantly more diversified HCV. Identification of a host system with
88 predominantly acute hepacivirus infection may enable new insights into the chronic infection
89 pattern associated with HCV.

90

91 **Importance**

92 The evolutionary origins of the human hepatitis C virus (HCV) are unclear. The closest
93 animal-associated relative of HCV occurs in horses (equine hepacivirus, EqHV). The low

94 EqHV genetic diversity implies a relatively recent acquisition of EqHV by horses, limiting
95 the time span for potential horse-to-human infections in the past. Horses are genetically
96 related to donkeys and EqHV may have co-speciated with these host species. Here, we
97 investigated a large panel of donkeys from various countries using serologic and molecular
98 tools. We found EqHV to be globally widespread in donkeys and identify potential
99 differences in EqHV infection patterns, with donkeys potentially showing enhanced EqHV
100 clearance compared to horses. We provide strong evidence against EqHV co-speciation and
101 for its capability to switch hosts among equines. Differential hepacivirus infection patterns in
102 horses and donkeys may enable new insights into the chronic infection pattern associated
103 with HCV.
104

105 **Introduction**

106 Hepatitis C virus (HCV) is a major human pathogen infecting approximately 140 million
107 people worldwide (1). HCV belongs to the genus *Hepacivirus* that comprises 7
108 geographically distinct genotypes which likely evolved over considerable time spans (2-6).
109 The evolutionary origins of HCV have remained obscure (6). Recent studies identified
110 numerous hepaciviruses (HVs) in bats, rodents, monkeys and peri-domestic animals (7, 8).
111 Considering the absence of HCV-related viruses in higher primates (9), as well as the
112 existence of genetically diversified nonprimate HVs, mammals other than primates may have
113 shaped primordial HCV evolution (10). The lack of co-segregation of HVs with mutually
114 related animal hosts, as well as the detection of potential recombination events between some
115 HV lineages suggest low barriers against cross-host transmission (10-13). However, whether
116 any of the animal species known to carry HVs represents a direct reservoir for HCV is
117 unclear (14).

118 The equine HV (EqHV, originally described as canine HV and subsequently as nonprimate
119 HV) (7, 8), constitutes the closest animal-associated relative of HCV among the HVs known
120 so far (7, 13). Sporadic infections of dogs (15-17) support a broad host range of EqHV that
121 may have enabled infection of humans with EqHV in the past. Transmission may have been
122 aided by close contact of humans and horses since the domestication of horses about 5,500
123 years ago (18). However, the strikingly low genetic variation of EqHV in horses suggests a
124 rather short evolutionary history (6), with limited opportunity for horse-human transition.

125 The genus *Equus* comprising all contemporary horses, donkeys and zebras likely originated
126 about 4.5 million years ago (19). Detection of EqHV homologues in equine sister species
127 may aid elucidating the evolutionary history of this HV. The globally most widespread
128 equine beyond domestic horses (*Equus ferus caballus*, ca. 59 million heads) is the
129 domesticated donkey (*E. asinus asinus*, ca. 44 million heads, according to the Food and

130 Agriculture Organization of the United Nations (FAO), FAOSTAT 2014 database). Donkeys
131 have been tested for HV in limited numbers, such as 116 donkeys from the UK (17, 20), 30
132 mules and 5 donkeys from Brazil (21), 8 mules and 6 donkeys from China (22), as well as a
133 commercially available donkey serum from the U.S. (23), all with negative results. Here we
134 investigated a considerably larger panel of donkey sera from various countries using
135 serologic and molecular tools. We found EqHV to be globally widespread in donkeys and
136 capable to switch hosts among equines.

137

138 **Materials and Methods**

139 **Sample collection**

140 Donkey sera were collected based on availability in France, Germany, Spain, Italy, Bulgaria,
141 Israel, Kenya, Mexico and Costa Rica from 1974-2016. Animal sera were stored at -20°C or -
142 80°C prior to analysis. Additionally, 53 mule samples were collected in Bulgaria in 2015.
143 Samples were either collected as part of routine examinations (Germany, Italy, Costa Rica
144 and France) or under permits issued by the responsible authorities. Permit numbers were:
145 Mexico; SICUAE FMVZ-UNAM F. García-Lacy 12042013, Kenya: IACUC 2015.8, Spain:
146 BOJA55-20/2012, Israel: KSVM-VTH/5_2013, Italy: Protocol #45/2013/CEISA/COM,
147 Bulgaria: FVM 15/15. Host designations were assessed for all EqHV RNA-positive
148 specimens from France from 1979 by characterization of the mitochondrial COI gene as
149 described before (24).

150

151 **Luciferase immunoprecipitation system (LIPS)**

152 All samples were analyzed for the presence of anti-NS3 antibodies by the previously
153 described LIPS (25). Briefly, sera were diluted 1:10 in buffer A and incubated for one hour
154 on a rotary shaker. Renilla-NS3 fusion proteins were expressed in Cos1 cells and 1×10^7

155 relative light units (RLU) were added per well to the diluted sera in a 96-well plate. After
156 incubation for one hour on a rotary shaker, antibody-antigen complexes were
157 immunoprecipitated by A/G beads and the RLU were determined. Each sample was
158 measured in duplicate wells. The cutoff was calculated by the mean values of wells
159 containing only buffer A, the Renilla-NS3 fusion protein and A/G beads plus three standard
160 deviations as described previously (25). A positive control containing anti-EqHV antibody-
161 positive horse serum was included in each run.

162

163 **Detection of EqHV RNA**

164 For the detection of hepaciviral RNA a hemi-nested RT-PCR assay targeting the 5'-UTR was
165 developed based on all available EqHV 5'-UTR sequences. Primer sequences were HCV-
166 F150, GSWSCYYCYAGGICCMCCCC; HCV-R371,
167 CTCRTGIISYAIGGTCTACRAGRCC; HCV-R342,
168 GGIGCICTCGCAAGCRYGCCYATCA (I=Inosine, S=C/G, W= A/T, Y=C/T, M=A/C,
169 R=A/G). Limits of detection were determined as the number in probit analyses conducted
170 with SPSS V23 (IBM, Ehningen, Germany) using 8 replicates per RNA concentration as
171 described previously (26). The 95% lower limit of detection of the EqHV 5'-UTR assay was
172 5.7×10^2 RNA copies per reaction (range, 3.8×10^2 - 1.2×10^3), which was well below the
173 commonly observed viral loads in EqHV-infected horses (27). The HV NS3-based assay was
174 described previously (11). Cross-tables were calculated using EpiInfo V7
175 (<http://www.cdc.gov/epiinfo/index.html>) and an online tool
176 (<http://quantpsy.org/chisq/chisq.htm>). Sequencing of the complete EqHV polyprotein genes
177 was performed by amplifying genome-spanning islets with degenerate broadly reactive
178 oligonucleotides as described previously (11). Viral loads were determined by strain-specific
179 quantitative real-time RT-PCR (oligonucleotide sequences available upon request) with

180 photometrically quantified *in vitro* cRNA transcripts used for calculation of the standard
181 curve as described previously (11).

182

183 ***In silico* analyses**

184 Statistical analyses were done using SPSS V23 (IBM, Ehningen, Germany). Sequences were
185 aligned with MAFFT (Geneious 6.1.8). Maximum likelihood phylogenetic analyses were
186 calculated in MEGA6 (28) and RAxML (29) using a general time reversible model with a
187 discrete gamma distribution and a proportion of invariable sites, and 1,000 bootstrap
188 replicates. To estimate branch lengths in synonymous and non-synonymous substitutions per
189 site, a codon substitution model was applied in HypHy (30) that allows for branch-specific
190 synonymous and non-synonymous substitution rates (31). PAML (32) was used to fit a codon
191 substitution model that allowed for a different non-synonymous/synonymous substitution rate
192 ratio (ω) on the branches leading to the two donkey HV common ancestors as compared to
193 the ω on the remaining branches (33). In addition, we used BUSTED (34) to search for gene-
194 wide evidence of episodic positive selection along the branches leading to the donkey virus
195 clades, and FUBAR (35) to identify site-specific selection patterns, both implemented in
196 HypHy. Root-to-tip divergence was plotted against sampling time using TempEst (36). Mean
197 folding energy differences (MFED) were calculated using SSE V1.2 as described previously
198 (12).

199

200 **GenBank sequence accession numbers**

201 All polyprotein gene sequences generated in this study were submitted to GenBank under
202 accession numbers KT880191-KT880193, and KX421286-KX421287.

203

204 **Results**

205 **Wide-reaching exposure of donkeys to EqHV**

206 Donkey sera (n=829) were collected in five European countries (Germany, Spain, Italy,
207 Bulgaria and France), as well as in Asia (Israel), Africa (Kenya), and Latin America (Costa
208 Rica and Mexico) between 1974 and 2016 (**Table 1**). For three countries (France, Germany,
209 Italy), sampling was conducted in multiple years and details of annual sample characteristics
210 in these countries are displayed in **Table 2**. Additionally, 53 mule sera were sampled in
211 Bulgaria in 2015. All 882 donkey and mule sera were analyzed for the presence of antibodies
212 against the viral NS3 domain by a luciferase immunoprecipitation system (LIPS) (25, 27).
213 Three sampling sites (Israel, Kenya and Costa Rica) showed no serologic evidence for EqHV
214 infection, whereas all other countries yielded positive test results (**Figure 1A**). As shown in
215 **Table 1** and **Figure 1B**, seroprevalence rates ranged between 8.1 and 10.7% in Germany,
216 Spain and Mexico. Seroprevalence rates in Italy and Bulgaria were significantly higher at
217 40.0-56.7% (corrected $\chi^2=62.8$ and $\chi^2=109.1$, $p<0.0001$ for Italy and Bulgaria compared to all
218 other countries, respectively). Furthermore, within a specific country the seroprevalence rates
219 varied between sampling years and hinted at the occurrence of focal EqHV epidemics, e.g.,
220 leading to 100% of EqHV-seropositive animals in Italy in 2015 (**Table 2**). However, the
221 underlying factors responsible for the variations in seroprevalence are unknown. LIPS signal
222 intensities from seropositive donkeys were comparable to those from seropositive horses,
223 suggesting validity of the assay used for testing (**Figure 1B**). Female donkeys were
224 significantly more likely to be seropositive than male donkeys (35.0 vs. 28.0%; corrected
225 $\chi^2=4.1$, $p=0.044$; Risk ratio, 1.25 (lower and upper bounds, 1.01-1.54); **Table 1**).
226 Seroprevalence increased significantly with animal age from 20.7% in young animals (0-5
227 years of age) to 55.5% in older animals (25-30 years) (**Figure 1C**).

228

229 Molecular detection of EqHV in donkeys

230 To allow sensitive molecular detection of EqHV genetic variants in donkeys, all samples
231 were tested using two different nested RT-PCR assays. The first assay targeted specifically
232 the EqHV 5'-untranslated region (5'-UTR) commonly used for HV detection (26) and a
233 second assay targeted the NS3 domain that is more conserved among diverse HVs than the
234 5'-UTR (11). One donkey from France (sampled in 1979, age and gender unknown), one
235 donkey from Bulgaria (sampled in 2015, a 10-year-old male) and one mule from Bulgaria
236 (sampled in 2015, a 16-year-old female) tested positive for EqHV RNA using the 5'-UTR-
237 based assay (0.3% of all 882 donkey and mule sera). No additional specimen tested positive
238 for HVs using the NS3-based assay, arguing against infection of donkeys with diverse HVs
239 beyond EqHV.

240

241 Comparison of EqHV infection patterns between equine species

242 Our data enabled comparisons of EqHV infection patterns between donkeys and horses. First,
243 viral loads, which are a quantitative marker of virus replication, were similar between equine
244 host species infected with EqHV. Viral loads in the RNA-positive specimens from this study
245 ranged from 8.4×10^5 to 3.7×10^7 genome copies/ml of serum, as determined by strain-specific
246 real-time RT-PCR assays. These viral loads were similar to viral loads observed in horses
247 (20, 23, 27), suggesting similar infection intensities in both equine species. Furthermore, the
248 detection of viral RNA at comparable loads in the French sera sampled in 1979 and Bulgarian
249 sera sampled in 2015 implicated suitability of the non-recently sampled specimens for viral
250 RNA detection.

251 Viral clearance is typically delayed in HV infection, including infection with EqHV in horses
252 (7). In our study, three serial individual specimens taken at different time points over two
253 weeks (May-June 1979) were available from the RNA-positive donkey sampled in France.

254 All three specimens as well as both individual specimens from Bulgaria tested positive both
255 for EqHV antibodies (indicated as red dots in **Figure 1B**) and RNA, providing evidence
256 against immediate antibody-mediated EqHV clearance in donkeys. However, the co-
257 occurrence of viremia and antibodies as a sign of delayed clearance was apparently much
258 lower in donkeys at 1.1% (3 of 278 antibody-positive animals) than in horses at 2-30% (17,
259 20, 25, 27, 37, 38).

260 Predominantly acute resolving infections were compatible with a generally lower RNA
261 detection rate in donkeys than in horses. Combining all available data from previous studies
262 on horses (17, 20-23, 25, 27, 37, 38), 148 of 2,172 horses tested positive for EqHV RNA
263 (6.8%, range 0.9%-35.5%), compared to only 3 of 1,047 donkeys or mules when combining
264 the data from this study with previous studies (17, 20-22) (0.3%; corrected $\chi^2=65.9$,
265 $p<0.00001$). The low number of RNA-positive donkeys could not be explained by a
266 putatively low exposure of donkeys to EqHV, since seroprevalence in donkeys was high at
267 28.3% (278 of 982 donkeys combining this and the only previous serological study (17)),
268 although still significantly lower than in horses at 34.9% (469 of 1,343 horses from all
269 previous studies performing serological analyses; corrected $\chi^2=11.1$, $p<0.0009$). The EqHV
270 seroprevalence increased with the age of donkeys, which was comparable to a study on
271 EqHV in German horses (27), but contrary to another study on EqHV in Japanese horses
272 (38). Finally, female donkeys were more likely to be seropositive for EqHV than male
273 donkeys (35.0 vs. 28.0%; corrected $\chi^2=4.1$, $p=0.044$; Risk ratio, 1.25 (lower and upper
274 bounds, 1.01-1.54)). A similar distribution was not observed for horses in two previous
275 studies, one showing no gender-associated differences and another one showing a higher
276 EqHV burden in male horses (27, 38).

277 Next, we investigated the clinical relevance of EqHV infection in donkeys by determination
278 of aspartate aminotransferase (AST, reference value <536 units (U)/L), gamma-glutamyl

279 transferase (γ GGT, <69 U/L) and glutamate dehydrogenase (GLDH, <8.2 U/L) levels in
280 serum of all Bulgarian donkeys (n=201) as markers of liver damage. As depicted in **Figure**
281 **1D**, liver enzymes concentrations were mainly within the reference range (39) and were
282 comparable between the seropositive and seronegative groups, including the RNA-positive
283 animals (given in color in **Figure 1D**), which is in line with the reported subclinical course of
284 infection in horses.

285

286 **Cross-species transmission of EqHV**

287 The full viral polyprotein genes were determined for all donkey EqHV strains, including
288 those from the three serial bleedings from the French donkeys, and those from the Bulgarian
289 donkey and mule. The polyprotein genes encompassed 8,832 nucleotides from the French
290 donkey EqHV strain, as well as 8,835 and 8,841 nucleotides from the Bulgarian donkey and
291 mule, respectively. Polyprotein length and organization was identical in all cases to that
292 observed before in EqHV from horses with presence of all typical domains in the order C-E1-
293 E2-p7-NS2-NS3-NS4A/NS4B-NS5A/NS5B. Maximum Likelihood (ML) phylogenetic
294 reconstructions based on the complete polyprotein gene were highly robust, as suggested by
295 high bootstrap support for clusters based on 1,000 replicates. In these ML phylogenetic
296 reconstructions, the novel donkey HVs from France and Bulgaria formed two distinct viral
297 lineages that were not monophyletic. In addition, these donkey HV lineages were interspersed
298 between EqHV from horses and did not cluster in sister relationships to EqHV strains from
299 horses (**Figure 2A**). The close phylogenetic relationship between EqHV strains from horses
300 and from donkeys or mules was compatible with a narrow genetic distance of only 1.8-2.5%
301 of the translated polyprotein genes of these strains. Of note, even upon inclusion of the novel
302 donkey viruses, the EqHV patristic distance was only 6.2% on amino acid level in the
303 translated polyprotein gene, compared to 33.1% within HCV (calculated using 189 genotype

304 1-7 reference sequences from the Los Alamos National Laboratory, <http://hcv.lanl.gov>.
305 However, most of the previous studies on EqHV in horses characterized only short regions of
306 the viral genome. Therefore, we repeated ML reconstructions using different datasets aiming
307 at inclusion of the complete available EqHV genetic diversity without losing too much
308 genetic information. As expected, statistical support for grouping of basal and intermediate
309 nodes was low for the partial NS3 (*helicase/protease*) and NS5B (*RNA-dependent RNA*
310 *polymerase*) domains commonly analyzed in EqHV studies. However, these reconstructions
311 resulted in similar phylogenies as shown for the complete polyprotein sequences with regard
312 to the phylogenetic relationships between EqHV strains from donkeys and horses (**Figure**
313 **2B-D**).

314 To investigate whether potential cross-species transmission was associated with molecular
315 adaptation, we tested for differential selection among horse and donkey EqHV lineages using
316 codon substitution models that allow for varying non-synonymous/synonymous substitution
317 rate ratios (dN/dS) among branches (33). Branches leading to the two common ancestors of
318 donkey EqHV strains showed a lower dN/dS ratio (0.02) compared to the dN/dS ratio among
319 all other branches in the complete genome data set (0.04), indicating no detectable episodic
320 adaptive signal underlying the transmission of EqHV strains from horses to donkeys.
321 Identical results were obtained for the dataset encompassing the full NS3, for which a larger
322 number of horse EqHV sequences were available (**Figure 2B**), with a dN/dS ratio of 0.0036
323 in branches leading to donkey EqHV strains compared to 0.0128 among other branches. An
324 analysis using BUSTED confirmed the absence of any signal of gene-wide episodic
325 diversifying selection along the branches leading to the two donkey clades. A FUBAR
326 analysis to identify site-specific selection only indicated two positively selected sites in the
327 complete polyprotein evolutionary history, which do not appear to be related to equine-to-
328 donkey adaptation because the donkey viruses do not share a particular amino acid residue on

329 those positions. In conclusion, the dN/dS ratios suggested that no host adaptation is needed
330 for the mutual infection of horses and donkeys with EqHV.

331

332 **Intra- and inter-host EqHV evolution**

333 In order to determine EqHV intra-host evolutionary patterns, the complete polyprotein gene
334 sequences of the three serial bleedings available from the French donkey were analyzed.

335 Intra-host variability within this viral gene spanning 8,832 nucleotides was 0.17% (15
336 substitutions) over two weeks (between May 23rd and June 6th, 1979; **Figure 3A**). Similar to

337 HCV, most mutations and in particular the majority of non-synonymous mutations occurred
338 in the antigenic E2 envelope protein (40), consistent with immune pressure influencing

339 EqHV evolution in the infected animal. However, the majority of the observed mutations did
340 not map to the N-terminal hyper-variable E2 region described for HCV (40), but accumulated

341 in the C-terminal region of the E2 gene. To investigate whether indeed EqHV generally
342 differs from HCV in the distribution of non-synonymous mutations in the E2 gene, the

343 homologous domains of 12 EqHV strains infecting horses were analyzed. As shown in
344 **Figure 3B**, EqHV strains infecting horses were similar to HCV in that 72 non-synonymous

345 mutations accumulated in the N-terminal region of E2, compared to only 28 non-synonymous
346 mutations in the C-terminal region. The different pattern observed in the EqHV-infected

347 donkey is thus likely due to the small dataset available, but potential differences of genomic
348 variability among EqHV hosts cannot be excluded at this point. Finally, reversion of two

349 mutations was detected across the serial bleedings (in the viral E2 and NS2 domains, **Figure**
350 **3A**), which again is similar to intra-host evolution patterns observed in HCV (41). The

351 predicted similarities in EqHV and HCV evolutionary patterns in combination with the low
352 EqHV patristic distance suggested a limited time of EqHV evolution in equines compared to

353 HCV in humans.

354 However, viral evolution may be limited by non-coding constraints such as genome-scale
355 ordered RNA structures (GORS). Albeit the level of predicted mean folding energy
356 differences (MFEDs, a measure of GORS) across the polyprotein-coding region was slightly
357 higher in donkey EqHV strains than the mean MFEDs within horse EqHV strains, the overall
358 EqHV MFED patterns showed similarities between both equine species in terms of the
359 presence and the extent of predicted stem-loops (**Figure 3C**). The overall levels of MFEDs
360 ranging up to 11.5% in our analyses were comparable to previous analyses of EqHV (15, 25)
361 and higher than the 8.5% described before for human HCV (42), which may imply a stronger
362 impact of GORS on EqHV than on HCV evolution (6). However, it seems unlikely that
363 GORS alone can account for the drastic differences between EqHV and HCV genetic
364 diversity.

365

366 **Lack of temporal signal in EqHV**

367 The donkey HVs sequences from 1979 represent the oldest EqHV strains described so far. In
368 order to investigate if these sequences could serve to calibrate the molecular clock of EqHV
369 evolution, root-to-tip distances were analyzed as a function of sampling time. To further
370 investigate if the temporal signal in EqHV was potentially influenced by evolutionary
371 pressure, root-to-tip distances were compared for complete polyprotein gene trees comprising
372 only non-synonymous (NS) or synonymous (S) substitutions (**Figure 3D**). The complete
373 polyprotein-based tree, as well as the trees with branch lengths re-estimated in either NS or S
374 substitutions lacked a molecular clock signal, as visualized by plotting root-to-tip divergence
375 against year of sampling (**Figure 3E**). Of note, lack of temporal signal upon inclusion of the
376 1979 donkey EqHV strains was consistent with the apical phylogenetic position of two EqHV
377 strains sampled from horses in 1997 and 1998 (20) (shown in cyan in **Figure 2C** and **2D**).
378 Unfortunately, only a partial NS3 sequence is available for the 1997 EqHV and only a partial

379 NS5B sequence for the 1998 EqHV strain, preventing their inclusion in our temporal
380 analyses.
381
382

383
384

Discussion

385 In this study we describe wide-reaching infection of donkey populations with EqHV and
386 analyze two divergent donkey EqHV lineages from contemporary and non-contemporary
387 samples.

388

389 If EqHV existed with donkeys for prolonged time spans, one could expect that donkeys
390 globally would show signs of infection. However, although infection with EqHV was
391 widespread and frequent according to our data, three populations in Kenya, Israel and Costa
392 Rica were entirely seronegative. Although this may be linked to the relatively smaller sample
393 sizes (n=15-44), some seropositive animals could be expected in these populations given the
394 8.1-56.7% seroprevalence in other donkey populations. Absence of EqHV infection in these
395 three populations is consistent with the absence of serological signs of EqHV infection in 100
396 English donkeys (17). The most parsimonious explanation is that EqHV was neither present
397 in the founders of these populations, nor introduced subsequently. Alternative explanations
398 include the extinction of EqHV in these populations together with their hosts. However, the
399 subclinical course of infection of EqHV suggested by the high seroprevalence rates in
400 animals of all ages and the limited clinical impact of EqHV on experimentally infected horses
401 (27) do not support high health costs of EqHV infection in donkeys.

402 Although the transmission routes of EqHV remain unclear, parenteral transmission is the
403 most likely route based on *in vivo* infection experiments and comparisons to HCV (27, 43).
404 Our data support frequent horizontal transmission in EqHV-infected populations, potentially
405 aided by human interference, e.g., vaccination or transfusion by veterinarians (38). The
406 higher seroprevalence we found in female donkeys may be compatible with a relevant
407 occurrence of sexual transmission in EqHV. This would be different from HCV, for which
408 sexual transmission is very infrequent (44), and for which detection rates and viral loads are

409 much lower in semen than in blood (summarized in (45)). Hypothetically, the absence of
410 higher EqHV seroprevalence in female compared to male horses (27, 38) may be obscured by
411 anthropogenic intervention. Another factor aiding higher seroprevalence in female donkeys
412 may be putatively larger groups held together, compared to more solitary male donkeys. This
413 hypothesis would be consistent with recently described herd-specific EqHV strains from
414 horses in Germany suggesting focal horizontal and vertical transmission (46). Experimental
415 infections, comparative testing of horse and donkey semen and additional epidemiological
416 data from both equine species will be necessary to elucidate how EqHV and HCV
417 transmission modes may differ. Furthermore, the reason for the high variability of RNA-
418 positive EqHV infections in horses (17, 20, 25, 27, 37, 38) is not clear yet. The only factor,
419 which has been noticed so far is the race and attendance in equestrian sports, respectively (21,
420 22, 27, 38).

421

422 The genetic relatedness of donkeys and horses likely facilitated the cross-species
423 transmission events suggested by our data (47). Hypothetically, the similarities in the time of
424 domestication of horses and donkeys 5,000-6,000 years ago (18, 48) would have facilitated
425 host shifts between the two equine species. However, the geographically most relevant area
426 for the domestication of horses was likely the Eurasian steppe (18), compared to northeastern
427 Africa for donkeys (49), narrowing the time span of frequent co-occurrence of these two
428 species to more recent times. It would thus be interesting to analyze ancient donkey species
429 for evidence of ancestral EqHV strains, including the wild African ass (*E. africanus*), which
430 is an evolutionary old species that likely contributed to the development of the widespread
431 domestic donkey (50). However, only few individuals exist nowadays within this species
432 classified as *Critically Endangered* by the International Union for the Conservation of Nature
433 (IUCN).

434 Our phylogenetic evidence provides clear evidence against a potential co-evolutionary
435 relationship between EqHV and different equine hosts, which diverged millions of years ago
436 (18, 19). The recent evolutionary history of EqHV thus narrows the time window for putative
437 equine-to-human transmission in the past as an explanation for the origins of HCV (10). Of
438 note, absence of past EqHV infections of humans is consistent with absence of signs of
439 present EqHV infection in different human cohorts (17, 51, 52). A short evolutionary
440 association between equine hosts and EqHV is also consistent with the highly diverse HV
441 lineages found in the genetically related hosts belonging to the order Artiodactyla (cattle).
442 The perissodactylan and artiodactylan lineages clearly did not co-speciate with their hosts
443 (12), and whether both of them are the result of independent cross-species HV transmission
444 events or whether unique host associations can be found for either the perissodactylan or
445 artiodactylan lineage remains to be determined. Immediate experimental approaches include
446 testing of related host species, e.g., zebras for the Perissodactyla and livestock species like
447 sheep or goats for the Artiodactyla.

448

449 Lack of deep-branching monophyletic clusters of EqHV strains from different regions
450 compared to the existence of geographically distinct HCV genotypes (2) are compatible with
451 global virus admixture through human interference, i.e., transport of infected animals or
452 animal products over wide geographic distances. The observation of viral admixture in equids
453 is paralleled by the occurrence of closely related HVs in cattle in Ghana and Germany (12,
454 53). Probably, the distribution of cattle has undergone anthropogenic change in an extent
455 similar to that of equids. An unrestricted exchange of EqHV strains among horses and
456 donkeys suggested by our phylogenetic data is consistent with the inability to calibrate a
457 molecular clock using EqHV strains sampled in 1979. Of note, our results do not exclude that
458 a clock-like signal may have existed in EqHV ancestors that evolved prior to the viruses

459 analyzed in this study. Similarly, a 40-year interval may be generally insufficient to analyze
460 the EqHV molecular clock. Interestingly, although investigations of the HCV molecular
461 clock have met considerable difficulties (40), a recent study was able to reconcile phylogeny
462 and sampling dates of archived HCV strains from 1953 (54). An interval spanning several
463 decades is thus not generally unsuitable for HV molecular clock analyses. Although we
464 cannot exclude the existence of potentially more diverse EqHV lineages in donkeys, our large
465 sample reached almost half of that of the combined previous studies into horses and extended
466 all of the latter in geographic extent, suggesting robustness of our evolutionary
467 reconstructions. Limitations of our study that can be circumvented in future prospective
468 studies include inhomogeneous sampling across sites, lack of knowledge on medical
469 treatment and health status of donkeys, as well as their contact to horses.

470

471 Finally, EqHV infection patterns in horses and donkeys may differ in the potentially higher
472 ability of donkeys to clear EqHV infection. First hints at possible explanations originate from
473 strikingly different EqHV RNA and antibody detection rates between different horse breeds.
474 More frequent EqHV infection may be linked to the frequency of veterinary examinations,
475 since valuable race horses and thoroughbreds seem to be particularly often infected by EqHV
476 (21, 27, 38). Alternatively, differences in immune responses influencing viral clearance may
477 occur between different horse breeds, although a generally higher susceptibility to viral
478 infections in thoroughbreds is not supported by data on equine Influenza (55). However, our
479 data permit hypotheses on differential immune control of EqHV by different equine species,
480 since donkeys may differ in their immune capacity from horses more than horse breeds from
481 each other (56). Again, alternative explanations that remain to be explored include less
482 intense veterinary handling of donkeys than in more valuable horse species. Beyond
483 investigations of EqHV ecology, our data suggest a unique opportunity to comparatively

484 investigate hepaciviral pathogenesis in a natural host. Here, infection courses can be directly
485 compared by experimentally infecting horses and donkeys with identical EqHV strains,
486 without the need to conduct highly restricted experimental infections of chimpanzees with
487 HCV lacking the simultaneous infection of the human counterpart (57).

488

489 In conclusion, our study highlights the impact of evolutionarily guided investigations into
490 viral ecology and offers new possibilities to elucidate factors involved in the development of
491 chronic HV infections.

492

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509

510

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- 708

709 **Figure legends**

710 **Figure 1. EqHV infection patterns**

711 A) Anti-EqHV antibody (ab) detection depicted in pie charts (red=positive). Asterisks, origin
712 of the EqHV-RNA positive animals. B) LIPS ratios of control sera from horses and donkeys;
713 Bulgaria includes as well 53 sera from mules. EqHV-RNA positive donkey and mule sera are
714 indicated in red. All three positive sera from France originate from one animal, no
715 seroprevalence rate for this country is indicated due to the low sample size. Dotted line, cut-
716 off (16,249.2 Relative light units [RLU]). C) Seroprevalence rates in different age groups. D)
717 Aspartate aminotransferase (AST), gamma-glutamyl transferase (γ GGT) and glutamate
718 dehydrogenase (GLDH) were determined in the sera of Bulgarian donkeys. Sera are shown
719 according to their LIPS status and RNA-positive samples are given in orange and blue.

720

721 **Figure 2. Phylogenetic relationships of EqHV including the novel donkey hepaciviruses**

722 A) Maximum Likelihood (ML) phylogeny based on the nucleotide sequences encoding for
723 the complete EqHV polyprotein including the newly described donkey EqHV strains
724 (orange). Bootstrap values larger than 75% are depicted as filled circles. Taxon designations
725 indicate GenBank accession numbers, country and year of sampling. B-D) ML phylogenies
726 based on the complete NS3 (1,872 nucleotides), partial NS3 (293 nucleotides) and partial
727 NS5B (261 nucleotides), respectively. Cyan, non-contemporary strains from two horses.
728 Partial NS3 sequences of which less than 200 nucleotides were characterized were not
729 included in the analysis shown in panel C to avoid further loss of genomic information and
730 robustness of phylogenetic reconstruction.

731

732 **Figure 3. EqHV evolutionary patterns**

733 A) Analysis of EqHV polyprotein sequences from three consecutive samples of one EqHV
734 RNA-positive donkey sampled in France. Grey bars, synonymous substitutions, black bars,
735 non-synonymous substitutions. B) Right, Locations of non-synonymous mutations in the E2
736 genes of EqHV strains infecting horses. Left, ML phylogeny of the translated sequences as
737 before. C) Mean folding energy differences (MFED) for complete polyprotein sequences of
738 EqHV strains representing both donkey EqHV lineages and all available EqHV polyprotein
739 sequences shown in Figure 2A. D) Complete polyprotein ML phylogenies with branch
740 lengths re-estimated using either non-synonymous or synonymous substitutions. Bootstrap
741 values larger than 75% are depicted as filled circles. E) Root-to-tip divergence plots based on
742 ML trees shown in panel C and Figure 2A.
743

744 **Table 1. Sample characteristics**

Country	Sampling year	n	Ab+ (%)	Gender			Age [years] (%)					
				Jack	Jenny	Unknown	0-5	6-10	11-15	16-20	21-30	Unknown
France	1974/1979	2	1 (50.0)	-	-	1/2 (50.0)	-	-	-	-	-	1/2 (50.0)
Germany	2007/2008/ 2015	56	6 (10.7)	0/10 (0)	6/46 (13.0)	-	0/8 (0)	4/14 (28.6)	0/5 (0)	0/4 (0)	0/2 (0)	2/23 (8.7)
Spain	2011	86	7 (8.1)	3/38 (7.9)	4/44 (9.1)	0/4 (0)	2/32 (6.3)	3/27 (11.1)	1/11 (9.1)	0/10 (0)	1/2 (50.0)	0/4 (0)
Italy	2004-2015	350	140 (40.0)	14/52 (26.9)	125/286 (43.7)	1/12 (8.3)	42/140 (30.0)	48/108 (44.4)	21/48 (43.8)	20/38 (52.6)	9/16 (56.3)	-
Bulgaria	2015	201	114 (56.7)	69/113 (61.1)	45/88 (51.1)	-	3/12 (25.0)	23/36 (63.9)	33/58 (56.9)	23/46 (50.0)	29/39 (74.4)	3/10 (30.0)
Israel	2014	44	0 (0)	0/29 (0)	0/15 (0)	-	0/5 (0)	0/9 (0)	0/2 (0)	0/2 (0)	-	0/26 (0)
Kenya	2015	34	0 (0)	0/17 (0)	0/6 (0)	0/11 (0)	-	-	-	-	-	0/34 (0)
Mexico	2016	94	10 (10.6)	4/53 (7.5)	6/41 (14.6)	-	4/41 (9.8)	4/33 (12.1)	2/17 (11.8)	0/2 (0)	0/1 (0)	-
Costa Rica	2016	15	0 (0)	0/9 (0)	0/6 (0)	-	0/8 (0)	0/6 (0)	0/1 (0)	-	-	-
Total		882	278 (31.5)	90/321 (28.0)	186/532 (35.0)	2/29 (6.9)	51/246 (20.7)	82/233 (35.2)	57/142 (40.1)	43/102 (42.2)	39/60 (65.0)	6/99 (6.1)

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747 **Table 2: Annual donkey sample characteristics for France, Germany and Italy**
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Country	Sampling year	n	Ab+ (%)	Gender			Age [years] (%)					
				Jack	Jenny	Unknown	0-5	6-10	11-15	16-20	21-30	Unknown
France	1974	1	0 (0)	-	-	0/1 (0)	-	-	-	-	-	0/1 (0)
	1979	1	1 (100.0)	-	-	1/1 (100.0)	-	-	-	-	-	1/1 (100.0)
Germany	2007	39	5 (12.8)	0/9 (0)	5/30 (16.7)	-	0/7 (0)	4/13 (30.8)	0/5 (0)	0/3 (0)	0/2 (0)	1/9 (11.1)
	2008	3	0 (0)	0/1 (0)	0/2 (0)	-	0/1 (0)	0/1 (0)	-	0/1 (0)	-	-
	2015	14	1 (7.1)	-	1/14 (7.1)	-	-	-	-	-	-	1/14 (7.1)
Italy	2004-2009	38	5 (13.2)	0/13 (0)	4/13 (30.8)	1/12 (8.3)	1/15 (6.7)	3/12 (25.0)	0/7 (0)	1/4 (25.0)	-	-
	2013	294	117 (39.8)	11/36 (30.6)	106/258 (41.1)	-	36/120 (30.0)	45/96 (46.9)	10/30 (33.3)	18/33 (54.5)	8/15 (53.3)	-
	2015	18	18 (100.0)	3/3 (100.0)	15/15 (100.0)	-	5/5 (100.0)	-	11/11 (100.0)	1/1 (100.0)	1/1 (100.0)	-

749

Figure 1

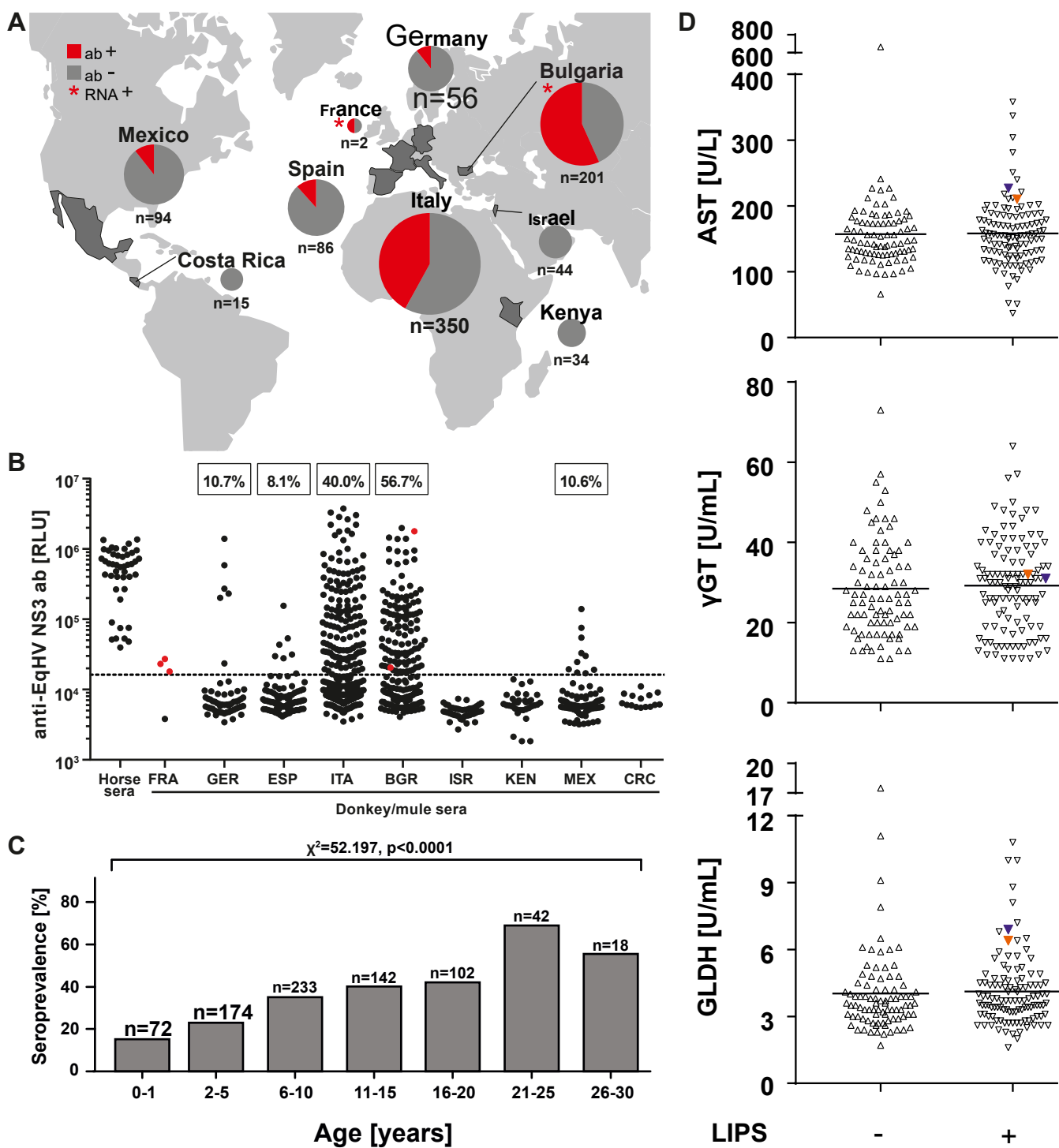


Figure 2

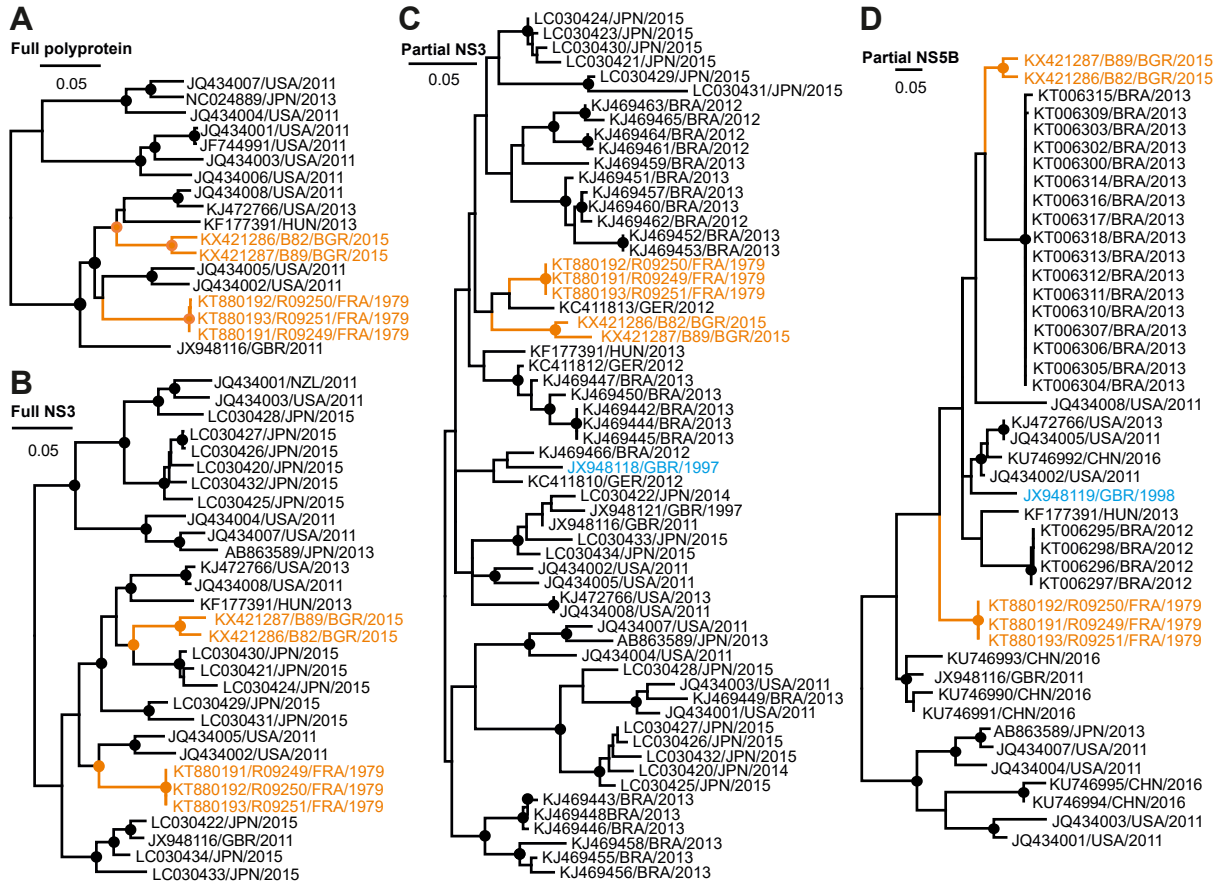


Figure 3

