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Short communication

Genetic diversity and evolution of Hare Calicivirus (HaCV), a recently identified lagovirus from *Lepus europaeus*

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ABSTRACT

First recognized as highly pathogenic viruses, hare lagoviruses belonging to genotype GII.1 (EBHSV) infect various *Lepus* species. Genetically distinct benign lagoviruses (Hare Calicivirus, HaCV) have recently been identified but few data have been available so far on these strains. The analysis of 199 samples from hunted hares collected throughout France allowed the detection of 20 HaCV and showed that they were widely distributed in this country. Ten HaCV capsid protein gene sequences were characterized. A first HaCV capsid protein structural model was proposed, revealing a global structure similar to that of a pathogenic GII.1 strain. The HaCV sequences showed an even higher genetic diversity than previously appreciated, with the characterization of two genotypes (GII.2, GII.3) and several additional putative genotypes. The most recent common ancestor for HaCV VP60 gene was estimated to be much older than that for GII.1 pathogenic strains. These results give new insights into the phylogenetic relationships of HaCV within the *Lagovirus* genus.

1. Introduction

The *Lagovirus* genus (*Caliciviridae* family) groups RNA viruses infecting European rabbits (*Oryctolagus cuniculus*) and several hare species (*Lepus* spp.) worldwide. The proposal for a classification system defined a single species *Lagovirus europaeus* (Le Pendu et al., 2017). Based on full-length capsid protein (VP60) gene sequences, the proposed genus was divided into genogroups GI and GII. GI was subdivided into four genotypes: GI.1 and GI.2 that include highly pathogenic viruses for both rabbits and hares related to rabbit hemorrhagic disease virus (RHDV), whereas GI.3 and GI.4 encompass benign viruses infecting rabbits. GII comprises one genotype (GII.1) that includes highly pathogenic viruses related to European brown hare syndrome virus (EBHSV). GII.1, GI.1 and GI.2 pathogenic viruses, first detected in 1980 in Sweden, in 1984 in China and in 2010 in France, respectively (Gavier-Widén and Mörner, 1991; Liu et al., 1984; Le Gall-Reculé et al., 2011a), cause acute necrotizing hepatitis.

The circulation of benign lagoviruses in leporid populations was suspected after identification of GI.1- or GII.1-seropositive samples predating the first outbreak descriptions of (Frölich et al., 2003; Robinson et al., 2002; Rodak et al., 1990). The first benign lagovirus, designated Rabbit Calicivirus (RCV), was described in 1996 in Italy (Capucci et al., 1996). Genetically distinct benign rabbit lagoviruses were subsequently characterized: GI.3/RCV-E1 (Moss et al., 2002; Le Gall-Reculé et al., 2011b), GI.4/RCV-E2 (Le Gall-Reculé et al., 2015) and GI.4/RCV-A1 (Strive et al., 2009). These lagoviruses target the duodenum and cause no mortality in experimentally infected rabbits (Le Gall-Reculé et al., 2011b; Strive et al., 2010). In comparison with rabbits, few data on benign lagoviruses are available in hares. The first presumably benign virus was detected from farmed hares in Italy in 2014 and its VP60 gene sequence was obtained (KR230102). This lagovirus, designated Hare Calicivirus (HaCV), was serologically and phylogenetically distinct from GII.1 viruses (Cavadini et al., 2015). It likely belongs to a new but not yet confirmed genotype (GII.2?; Le

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Pendu et al., 2017). Another HaCV strain was detected from an apparently healthy French wild hare in 2015. Its entire genome sequence (MH204883) showed a genomic organization typical of lagoviruses and 79% nucleotide identity with GII.1 viruses (Droillard et al., 2018). That study underlined the high genetic distance between the Italian and French HaCV VP60 sequences (15.3%). Recently, three phylogenetically distinct HaCV were characterized from apparently healthy Australian wild hares in 2016–2017. Genomic sequences (MK138383–MK138385; Mahar et al., 2019) shared between 74 and 78% nucleotide identity across sequenced regions and the authors suggested that they could belong to three putative genotypes.

We aimed at characterizing new HaCV from wild *Lepus europaeus* collected in France to improve our knowledge of the genetic diversity and evolutionary pattern within the *Lagovirus* genus. Ten new VP60 gene sequences were used to establish the phylogenetic relationships between hare lagoviruses and to estimate the substitution rate and TMRCA in capsid genes. We obtained the first structural model of a HaCV VP60 and compared it with that of a pathogenic hare lagovirus.

2. Materials and methods

Duodenum samples from 199 hunted and apparently healthy hares collected throughout France during the hunting season 2014–2015 and one liver from a dead hare collected in 2014 were analyzed. No animals were killed for the purpose of this study. Total RNAs were extracted from duodenum or liver samples as previously described (Le Gall-Reculé et al., 2011b). Screening for lagovirus RNA was performed from duodenum samples by one-step RT-PCR using U38 and L337 as primers (Le Gall-Reculé et al., 2011b) and SuperScript™ III One-Step RT-PCR with Platinum Taq HiFi (Invitrogen). For the liver sample, RNAs were reverse transcribed and amplified using the U38/EBHS9 PCR (Lopes et al., 2013). Complete VP60 genes were amplified by PCR or semi-nested PCR using a set of primers (Table S1). Sequencing of the PCR products, compilation of the consensus sequences and their genotyping were performed as described in Le Gall-Reculé et al. (2017).

For homology modelling of HaCV VP60, sequence alignment of selected templates and VP60 sequences from HaCV E15–219 (MH992074) and GII.1 O282/Sweden/1984 (KJ679522) was calculated from pairwise structure alignment (Align123 algorithm, Discovery Studio 2017, Dassault Systèmes BIOVIA, San Diego) and manually optimized (Fig. S1). Conditions for the structure model determination are indicated in the Fig. S2 caption.

Complete and nearly complete lagovirus VP60 sequences available in nucleotide databases were aligned with the characterized HaCV sequences using ClustalW (MEGA software version 7; Kumar et al., 2016). The phylogenetic relationships among the selected sequences were estimated using the maximum likelihood method (MEGA7). Pairwise nucleotide distance comparisons based on the p-distance model, complete deletion option, were conducted in MEGA7 with 25 HaCV VP60 sequences.

The rate of both nucleotide substitution per site and the time to most recent common ancestor (TMRCA) were estimated for HaCV and GII.1 VP60 genes by using the Bayesian Markov Chain Monte Carlo (MCMC) approach (BEAST package version 1.8.4; Drummond et al., 2012). Complete HaCV sequences were analyzed (19/25) and a selection of GII.1 sequences representing the genetic diversity was used.

3. Results and discussion

Twenty HaCV were detected from samples collected in different French regions revealing that they are well represented in *Lepus europaeus* populations. In addition to the French HaCV/E15–431 previously characterized (Droillard et al., 2018), ten VP60 genes were sequenced (MH 992067–MH992075, MN128592), which constitutes a significant contribution to the knowledge of the genetic diversity and distribution range of HaCV.

HaCV VP60 sequences were 1731 nucleotides long (576 aa long) except HaCV/E15–226 that was 1728 nucleotides long (575 aa long) due to a deletion of three nucleotides in frame (nt 1224–1226, aa 408). The average nucleotide genetic distance between the French HaCV and GII.1 was 25%. The closest rabbit lagovirus genotypes were GI.4 (28%), then both GI.1 and GI.2 (30%), and GI.3 (31%). Between themselves, the French HaCV sequences exhibited between 6.0% and 26.8% divergence (between 2.2% and 14.6% at aa level). Except HaCV/E15–226, the French sequences had a 17% average genetic distance with the Italian HaCV, 22% with the Australian HaCV-A2 and 26% with the Australian HaCV-A1 and HaCV-A3 sequences. The HaCV/E15–226 sequence exhibited an average genetic distance of 26% with the Italian HaCV and HaCV-A2 sequences, but 20% with the HaCV-A1 and HaCV-A3 sequences and the same deletion.

The HaCV/E15–219 VP60 was predicted to have the same structural organization as other lagovirus VP60 (Fig. S2) and comparison with that of EBHSV/O282/Sweden/1984 VP60 showed overall homology despite some differences especially in the flexible loops of the protrusion P2 subdomain.

The proposal for lagovirus taxonomy defines as a new genotype any well-supported phylogenetic group (bootstrap value > 70), gathering at least three VP60 nucleotide sequences from epidemiologically independent strains and with an average genetic distance between sequences > 15% with the other phylogenetic groups (Le Pendu et al., 2017). Thus, phylogenetic analyses based on the lagovirus VP60 sequences including the 15 HaCV previously characterized and the 10 HaCV from this study revealed that HaCV formed two genotypes, GII.2 and GII.3 (Fig. 1). Both were genetically distant from known lagovirus genotypes but were more related to GII.1. GII.2 was highly supported (bootstrap value 92) and included sequences of the 11 Italian HaCV and of seven French HaCV. GII.3 was highly supported (bootstrap value 100) and included the sequences of the Australian HaCV-A1 and HaCV-A3 with that of the French HaCV/E15–226. The three French HaCV/E15–219, HaCV/E15–304 and HaCV/E15–425 as well as the Australian HaCV-A2 remained unclassified (Fig. 1). However, the high genetic diversity displayed by the HaCV strains strongly suggests that more genotypes could later be defined including the four putative genotypes previously identified (Le Pendu et al., 2017; Mahar et al., 2019). In comparison with rabbit benign lagoviruses divided into two genotypes (GI.3, GI.4), the higher genetic diversity among hare lagoviruses might suggest that HaCV viruses evolved in hares during a longer time span than benign viruses did in rabbits. Of note, GII.3 contains two Australian and one French strains, confirming that the Australian HaCV do not cluster into a lineage separate from that of the European viruses (Mahar et al., 2019). These sequences shared the same residue deletion, a feature not found in any other lagovirus sequences. These findings suggest a common genetic origin of European and Australian HaCV strains and support the hypothesis of the introduction of HaCV in Australia with the introduction of HaCV-infected hares from Europe.

The MCC tree was performed with 19 HaCV and 20 selected GII.1 complete VP60 nucleotide sequences (Fig. 2). MCMC analysis revealed that the mean rate of evolutionary change in GII VP60 gene was 2.71×10^{-3} nucleotide substitutions per site per year (subs/site/year) with a variation across coalescent model of $1.98\text{--}3.43 \times 10^{-3}$ subs/site/year (95% HPD values). It was similar to the GII.1 and GI.1 VP60 genes and slightly higher than that of GI.2 (Table 1). Thus, the TMRCA for GII.1 VP60 gene was estimated in 1974 (1969–1980) (Table 2), consistent with the time of emergence of EBHS in Europe and validates the mean rate of evolutionary change determined in GII VP60 gene. The TMRCA for GII.2 and unclassified HaCV VP60 genes and for GII.3 VP60 gene were estimated before that of GII.1 (1879 and 1923, respectively; Table 2). With the current dataset, it is not possible to firmly conclude that GII.1 appeared after HaCV, but the characterization of GII.1 together with the first reports of outbreaks in 1980 strongly suggest that HaCV circulated earlier. The estimated TMRCA for HaCV and GII.1 VP60 genes (1813) also suggests that HaCV have evolved in hares on a

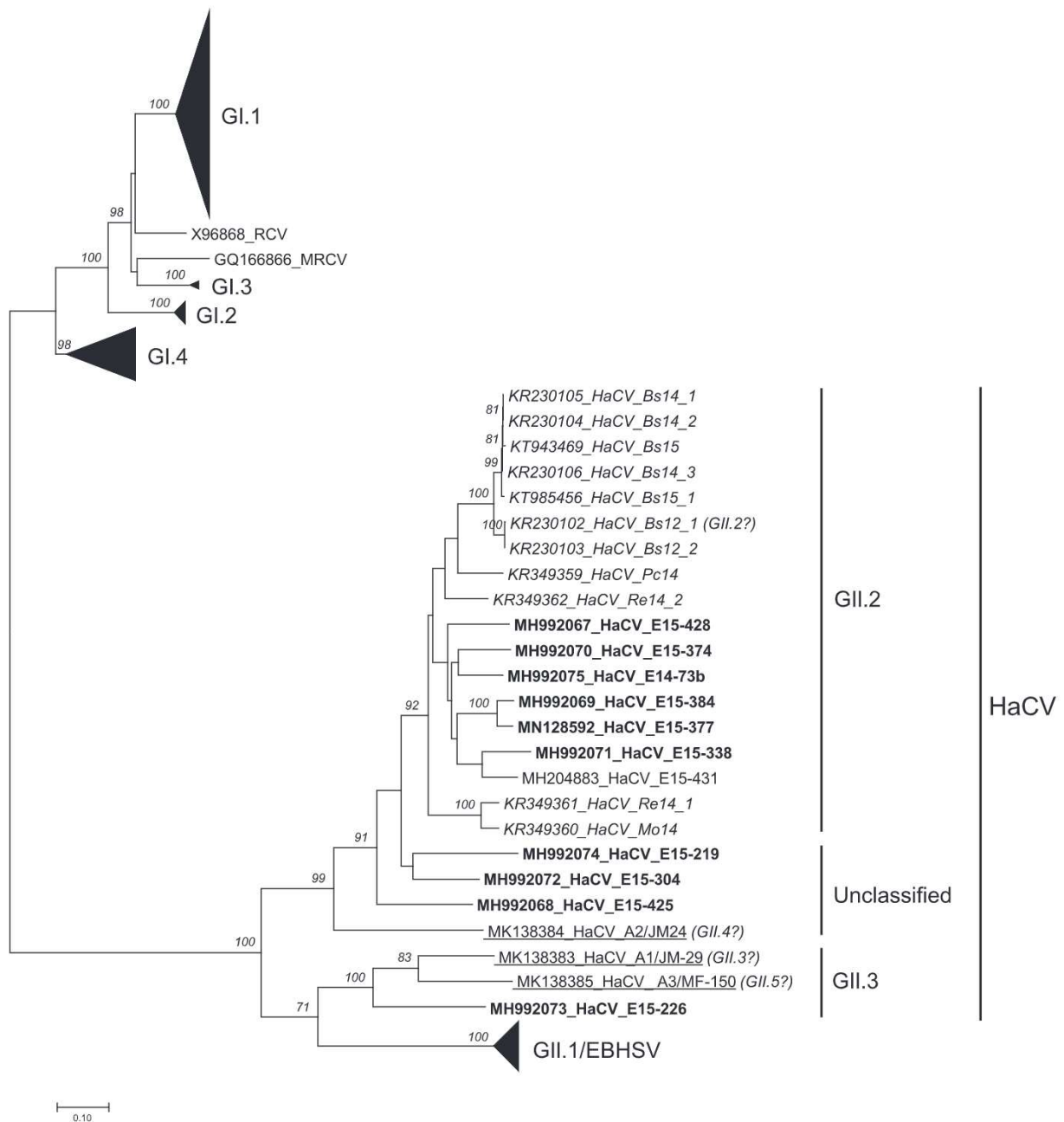


Fig. 1. Maximum likelihood phylogenetic tree of complete and nearly complete lagovirus VP60 gene nucleotide sequences ($n = 190$). The maximum likelihood method (MEGA software version 7; Kumar et al., 2016) was implemented with the GTR + Γ model of nucleotide substitution and a NNI branch-swapping. The Jalview2 software (Waterhouse et al., 2009) was used to reduce the number of available sequences in nucleotide databases (redundancy threshold of 98). The final dataset is 190 sequences. The tree was rooted using GI viruses. Significant bootstrap values greater than 70 for 1000 replicates are shown in italic after each node. Lagovirus genotypes GI.1–4 and GII.1 are represented as collapsed in order to reduce the tree size and highlight the HaCV sequences. French HaCV sequenced in this study are shown in bold, Italian HaCV in italics and Australian HaCV are underlined. The four putative HaCV genotypes (GII.2–4?) previously suggested by Le Pendu et al. (2017) and Mahar et al. (2019) are indicated in italics for the corresponding HaCV strains.

longer time span than GII.1 (Table 2). These results suggest that one unique event occurred before 1980, associated with appearance of pathogenic lagoviruses in hares. However, the current dataset is not sufficient to determine whether pathogenic lagoviruses were generated because of mutations of a benign virus and/or from a species jump as hypothesized by Esteves et al. (2015).

More HaCV VP60 sequences from a broader geographic range are needed to confirm the putative genotypes and improve the accuracy of the TMRCA analysis. Obtaining the complete genomic sequences of the characterized HaCV would help to understand their evolutionary history. Animal experiments are also required to ascertain the phenotype

of these HaCV viruses presently considered as benign.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2020.104310>.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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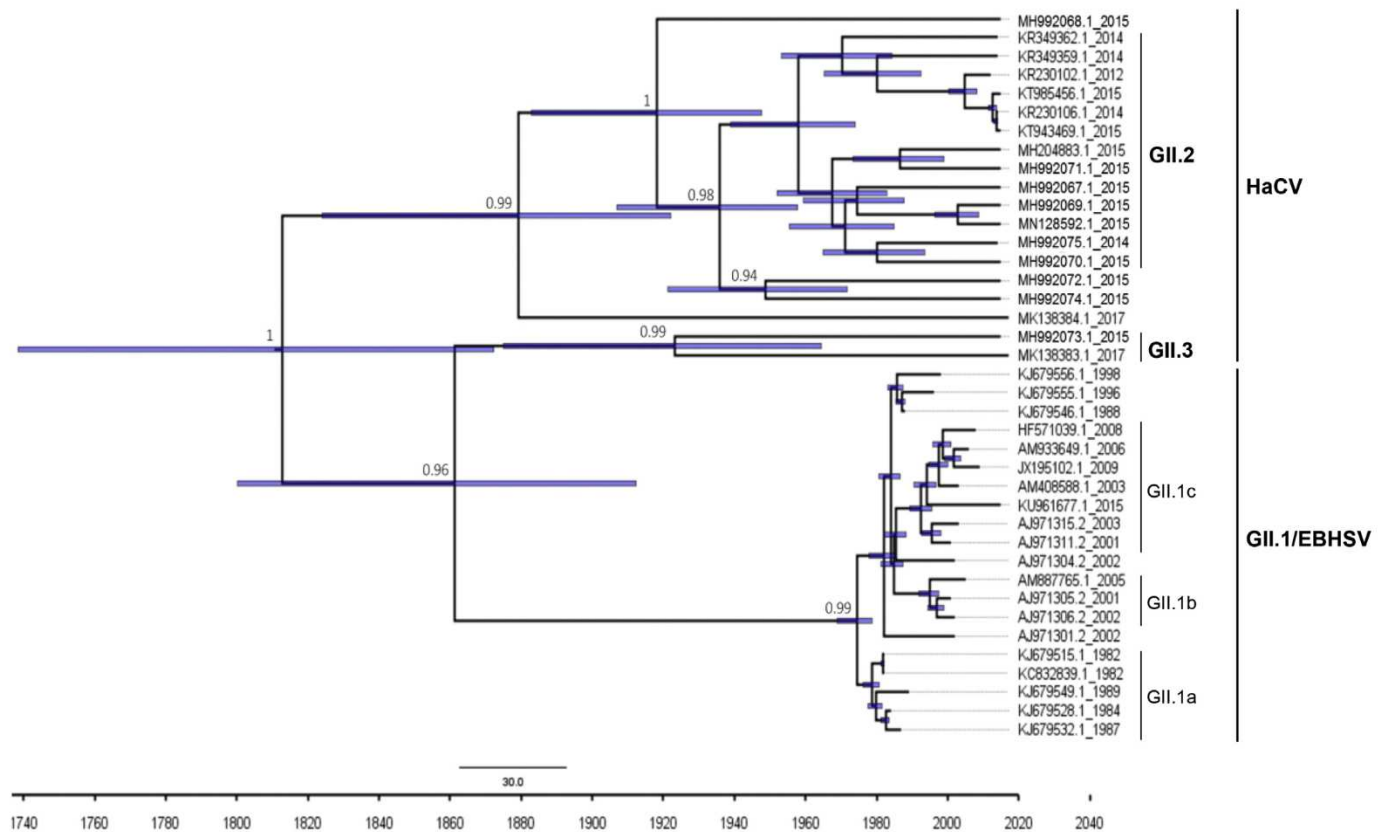


Fig. 2. Maximum Clade Credibility (MCC) tree of complete HaCV VP60 sequences ($n = 19$) and a selection of complete GII.1 sequences ($n = 20$) representing the genetic diversity (GII.1a-c). The time-scale (x-axis) is given in years. The node bars depict 95% HPD values on node height (age) and Bayesian posterior probability values > 0.9 are shown for major nodes. In all cases, tip times reflect the collection date of the sample. Relaxed (uncorrelated lognormal) molecular clocks and a substitution model using the Hasegawa, Kishino and Yano HKY + Γ + I substitution model with a different rate assigned to each codon position were used. Constant coalescent population size was used in all cases. MCMC chains were run for 100 million generations (assessed by using the TRACER program: <http://tree.bio.ed.ac.uk/software/tracer/>) with uncertainty in parameter estimates reflected in values of the 95% highest probability density (HPD). Maximum Clade Credibility (MCC) tree from all of the plausible trees created was obtained using the TreeAnnotator program with the first 10% trees removed as burn-in.

Table 1

Mean rates of evolutionary change determined for VP60 genes of lagovirus genotypes.

VP60 gene	Mean rate of evolutionary change (substitution/site/year)	95% highest posterior density (HPD) interval	Reference
GII	2.71×10^{-3}	$1.98\text{--}3.43 \times 10^{-3}$	This study
GII.1	3.00×10^{-3}	$2.60\text{--}3.60 \times 10^{-3}$	Lopes et al., 2014
GI.1	2.77×10^{-3}	$2.47\text{--}3.08 \times 10^{-3}$	Eden et al., 2015
GI.2	3.93×10^{-4}	$3.32\text{--}4.58 \times 10^{-4}$	Silvério et al., 2018
GI.4/RCV-A1	5.60×10^{-3}	$4.60\text{--}7.00 \times 10^{-3}$	Mahar et al., 2016

Table 2

Estimation of the time to most recent common ancestor (TMRCA) for GII VP60 genes.

VP60 gene	TMRCA	95% highest posterior density (HPD) interval	Bayesian posterior probability (BPP)
GII.1	1974	1969–1980	0.99
GII.2 and unclassified HaCV	1879	1824–1922	0.99
GII.3	1923	1876–1964	0.99
GII.3 and GII.1	1861	1800–1915	0.96
HaCV and GII.1	1813	1738–1872	1.00

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