


Widespread occurrence of the non-pathogenic hare calicivirus (HaCV Lagovirus GII.2) in captive-reared and free-living wild hares in Europe

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Abstract

The *Lagovirus* genus comprises both pathogenic viruses as European brown hare syndrome virus (EBHSV- GII.1) and rabbit hemorrhagic disease viruses (RHDV-GI.1 and RHDV2-GI.2), that principally infect European brown hares (*Lepus europeus*) and European rabbits (*Oryctolagus cuniculus*), respectively, causing severe necrotic hepatitis, spleen enlargement and disseminated haemorrhage. This genus includes also non-pathogenic agents, such as rabbit calicivirus (RCV-E1 – GI.3) and the non-pathogenic hare *Lagovirus*, provisionally named hare calicivirus (HaCV – GII.2). The latter had been identified for the first time in 2012 in the gut contents and faeces of healthy young hares raised in a breeding farm. In this study, we further investigated the presence of HaCV by testing the intestinal tract of 621 wild hares collected between 2010 and 2018 in Northern and Central Italy, and in 2011 in Austria, Germany and Spain. These wild hares were found dead for causes other than EBHS or were healthy hares shot during the hunting season. Forty-three out of 322 hare samples from Italy and 14 out of 299 samples from Austria and Germany were positive for HaCV-GII.2 by RT-PCR using universal primers for lagoviruses and primers specific for HaCV. Sequence analysis of the full capsid protein gene conducted on 12 strains representative of different years and locations indicated that these viruses belong to the same, single cluster as the prototype strain initially identified at the hares' farm (HaCV_Bs12_1). The relatively high level of genetic variation (88% nt identity) within this cluster suggests HaCVs may have been circulating widely in Europe for some time.

KEYWORDS

epidemiology, hare, Lagovirus, non-pathogenic, phylogenesis

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1 | INTRODUCTION

European brown hare syndrome (EBHS) and rabbit hemorrhagic disease (RHD) are two distinct diseases caused by lagoviruses that are responsible for acute and lethal hepatitis in lagomorphs. These diseases emerged around 1980 in northern Europe (Gavier-Widén & Mörner, 1991) (EBHS) and 1984 in China (Liu, Xue, Pu, & Quian, 1984) (RHD) and have similar epidemiological, clinical and pathological characteristics (Capucci, Scicluna, & Lavazza, 1991). The *Lagovirus* genus belongs to the Calciviridae family (Email ratification March, 2020; ICTV Virus Taxonomy, 2019; Wirblich et al., 1994). Although closely related, rabbit hemorrhagic disease virus (also called RHDV-GI.1, based on recently proposed genomic nomenclature) (Le Pendu et al., 2017) and EBHSV-GII.1 are classified as two viral species, being genetically and antigenically distinct (Capucci et al., 1991; Lavazza, Scicluna, & Capucci, 1996; Vinjé et al., 2019). These viruses also have strict host specificity in nature and under experimental conditions (Bergin et al., 2009; Lavazza et al., 1996). In 2010, a new RHDV strain, named RHDV2 (GI.2), that affected both farmed and wild rabbits, was described in France and Italy (Le Gall-Recule et al., 2013; Le Galle Reculé et al., 2011a) and subsequently reported in most European countries (reviewed by Neimanis et al., 2018). Based on phylogenetic analysis and the ability to infect hare species such as *Lepus capensis* (Puggioni et al., 2013), *L. corsicanus* (Camarda et al., 2014), *L. europaeus* (Le Gall-Reculé et al., 2017; Velarde et al., 2016) and *L. timidus* (Neimanis et al., 2018), RHDV2 should be more properly classified as a newly emerged virus and a new serotype, rather than a variant of the existing RHDV.

A third type of lagovirus, rabbit calicivirus (RCV), provisionally described as GI.5 by Le Pendu et al. (2017), was initially detected in Italy in 1996 in domestic rabbits (Capucci, Fusi, Lavazza, Pacciarini, & Rossi, 1996). This virus infects the intestinal tract without causing any clinical signs or macroscopic lesions. Additional non-pathogenic lagoviruses have subsequently been detected in domestic and wild rabbits in Europe (RCV-E1 - GI.3) (Le Gall-Reculé, Lemaitre, Briand, & Marchandau, 2015; Le Gall-Reculé et al., 2011b) and Australia (RCV-A1- GI.4) (Jahnke, Holmes, Kerr, Wright, & Strive, 2010; Strive, Wright, Kovaliski, Botti, & Capucci, 2010; Strive, Wright, & Robinson, 2009). These viruses are present at highest concentrations in the duodenum, are excreted in the faeces and are present in very low amounts in the liver (Strive et al., 2009). It has been speculated that the existence of a non-pathogenic EBHS-like virus in hares may provide a possible explanation for “unexpected” positive serological results obtained from brown hares and other *Lepus* species inhabiting areas where the disease has never been detected (i.e. Australia, South American and Central Africa) (Stott P., Trocchi V. and Capucci L., personal observations; Frölich, Kujawski, Rudolph, Ronsholt, & Speck, 2003; Lavazza et al., 1992).

We previously reported the first identification of a new lagovirus, HaCV_Bs12_1 (GB KR230102), in the faeces and intestines of captive-reared healthy hares (Cavadini et al., 2015, and manuscript in preparation). The new virus was repeatedly detected in healthy hares at a breeding hare farm in the Brescia province in Northern Italy, from 2012 until the farm's closure in 2016. Similar HaCVs were

subsequently described also in France (Droillard et al., 2018; Droillard et al., 2020) and Australia (Mahar et al., 2019). Genetic relationships, based on the complete capsid gene sequences, indicate that HaCV is phylogenetically distinct from all previously described members of the genus *Lagovirus* and represents a new genetic group (Cavadini et al., 2015; Droillard et al., 2018; Droillard et al., 2020; Le Pendu et al., 2017; Mahar et al., 2019).

The aim of the present study is to report the HaCV prototype strain's full genomic sequence (HaCV_Bs12_1) and describe the detection and partial genomic characterization of HaCV strains from wild hares collected between 2010 and 2018 in northern-central Italy, Germany, Austria and Spain. These data are important for clarifying the evolutionary history, genetic diversity and geographic distribution of HaCV, also in relation to other benign lagoviruses.

2 | MATERIALS AND METHODS

2.1 | Sampling

Wild hare samples ($n = 621$) were obtained between 2010 and 2018 from Northern and Central Italy (Lombardy, Emilia Romagna and Tuscany regions, including Pianosa Island) ($n = 322$), Germany (Bavaria and Pellworm Island, North Sea) ($n = 104$), Austria (Lower Austria) ($n = 106$) and Spain (Navarre) ($n = 89$). Samples were collected from three hare species: (a) brown hares (*L. europaeus*) ($n = 531$), 33 of which were of the subspecies *L. europaeus* var. *meridiei* from Pianosa Island (Tuscany Archipelago), (b) Italian hare (*L. corsicanus*) ($n = 1$) from Lucca province and (iii) Iberian hare (*L. granatensis*) ($n = 89$) from Spain.

Sampled hares were mostly hunted, with a few cases found dead due to causes other than EBHS during passive surveillance. Collection sites included open estates and closed/fenced “restocking” areas. Almost all samples were collected during hunting season (from mid-September to beginning of December). The samples on Pianosa Island were collected at the end of November. The age was not determined, but based on the sampling period, they were estimated to be all subadults or adults. The proximal duodenum was sampled during necropsies and immediately stored at -80°C .

2.2 | Extraction, detection and sequencing of viral RNA

Total RNA was extracted from 50–100 mg of duodenum disrupted using TissueLyser II (Qiagen, Hilden, Germany) and 1 ml of TRIzol reagent (Qiagen), according to the manufacturer's instructions. Viral genome detection was conducted by amplifying 3 μl of RNA using the One-Step RT-PCR Kit (Qiagen) with universal primers for *Lagovirus* Rab1/Rab2 (Strive et al., 2009) and/or with the primers HaCV-F/HaCV-R (Table 1). The amplification product was sequenced in both directions using the dye terminator method (Applied Biosystems)

TABLE 1 Primers used for virus identification and sequencing of HaCV

Primer	Sequence 5'-3'	Position nt ^a
<i>Primers for virus identification</i>		
HaCV-F	GGGCACCCAAACCACAGC	5,304–5,321
HaCV-R	AAYTGCATHCCACCHGCCCA	5,600–5,619
<i>Primers for amplification and sequencing the HaCV genome</i>		
EBHS5-F	CGACAGGAAGAGGATCGTCT	5,223–5,242
EBHS7-R	AAACCTGGGGCTGGACCAGC	6,137–6,121
EBHS-NP2-R	AGGGCTGGACCAACCAAAGTTG	6,132–6,110
EBHSV-NP1-F	CAGTCGACTGTTGAGTCCGCTT	5,952–5,974
EBHSV-1786-R	GCTCCAGCCAATGTTAGTCTCTAGA	7,032–7,009
HaCV-AF	ATTATGGCGGTTGCGTCGCG	1–20
HaCV-AR	CTCGGCAGCTGCGATCATGTC	622–643
HaCV-BF	GTTTGTGACCTTGCTGAACCGTG	512–536
HaCV-BR	CAATGCCAGGGGCACCTTTGAA	1,543–1,564
HaCV-CF	CCGCATTCTACAGGCGTGTTA	1,862–1,882
HaCV-CR	CGATTTGTCCAGCTGAAG	2,838–2,857
HaCV-DF	GGCCGAATAACAACATATGGG	3,513–3,534
HaCV-ER	GTCAGCAAGGATGTCAATGGC	4,528–4,548
HaCV-FF	GTTCCACCAAGAGGGGTTTGCC	4,630–4,652
HCV-50R	GATGCTGTGGTTGGGTGCCCG	5,303–5,324
HaCV-GF	GGCAGCTCAACTTTCCTCCGG	6,806–6,827
VP10-F	ATGTCTGAATTTCTAGGACTAAC	6,998–7,017
Oligo-dT adapter	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTT	/
Adapter	GACTCGAGTCGACATCG	/

^aNucleotide position based on the HaCV sequence KR230102.

and analysed with the software DNASTAR Lasergene Core Suite 10 (DNASTAR). The entire VP60 gene was amplified using two overlapping PCR products (a) EBHS5-F/EBHS7-R or EBHS-NP2R, and (b) EBHSV-NP1-F/ EBHSV-1786-R (Table 1) with the SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity (Life Technologies), and the products were then sequenced. Contig assembling and genome sequence analysis were carried out using Seqman NGen DNASTAR version 11.2.1 (DNASTAR). A maximum-likelihood phylogenetic analysis using MEGA 6 employing the GTR+G+I nucleotide substitution model (Tamura, Stecher, Peterson, Filipiński, & Kumar, 2013) was performed. Bootstrap analysis was performed using 1,000 replicates.

2.3 | Full genome sequence

The full genome sequence of HaCV_Bs12_1 (the first HaCV identified in Italy in 2012) was obtained using modifications of the method described by Victoria, Kapoor, Dupuis, Schnurr, and Delwart (2008). Briefly 160 mg of duodenum was homogenized with 2 ml of PBS1X using TissueLyser II (Qiagen), and the homogenate was sequentially filtered through 0.45- and 0.22-µm filters (Millipore). Filtered homogenate (200 µl) was incubated with 14U DNaseTURBO (Life Technologies) and 20U RNaseONE (Life

Technologies) at 37°C for 30 min to remove the host genome, followed by the addition of 800 µl of TRIzol (ThermoFisher Scientific) for RNA extraction. Viral cDNA synthesis was conducted by incubating viral RNA with 100 pmol of primer K-8N (GAC CAT CTA GCG ACC TCC CAN NNN NNN N) with Superscript II, according to the manufacturer's instructions (Life Technologies). To generate products with primer K-8N fixed on the opposite strand of cDNA, a single round primer extension was performed using a Klenow fragment and K-8N primer, followed by synthesis of a second strand using Klenow enzyme (Life Technologies). PCR on extension products was performed using 5 µl of the reaction described above with primer K-8 (GAC CAT CTA GCG ACC TCC AC) and Platinum Taq DNA polymerase (Life technologies). Products were distinguished using agarose gel electrophoresis followed by purification. After quantification, the DNA was sequenced with a MiSeq Instrument (Illumina Inc.) using Miseq Reagent Kit v2 (Illumina Inc.) in a 250-cycle paired-end run. Data were assembled de novo with the NextGen DNASTAR (DNASTAR Inc., WI, USA) application and were analysed using Lasergene Package software (Ver 12.0) (DNASTAR Inc.). Based on the sequence reads, gaps were filled by RT-PCR using the primers reported in Table 1. To obtain the 3'-end of the genome, cDNA synthesis was performed using oligo-dT-adaptor primer followed by PCR using HaCV-GF/Adapter primers (Frohman, Dush, & Martin, 1988) (Table 1).

2.4 | Screening of hares for lagovirus and sequence analysis of the vp60 gene

All samples from Italy, Germany, Austria and Spain were screened by RT-PCR using both the universal primers for *Lagovirus* Rab1/Rab2 (Strive et al., 2009) and the specific primers for HaCV. The latter was based on sequences for HaCV_Bs12_1 and all other strains identified at the farm study site (Cavadini et al., 2015; manuscript in preparation).

3 | RESULTS

3.1 | Full genome sequence

The length of the full genome of the first isolate of HaCV_Bs12_1 is 7,423 nucleotides (nt) (CDS), and it is organized into two predicted open reading frames (ORFs): ORF1 is 7,005 nt long and codes for a putative 2,334 amino acid (aa) polyprotein, whereas ORF2 is 342 nt long with a 3 nt deletion (193–195 nt) when compared to EBHSV (NC002615) and HaCV (MH204883) and encodes for a putative 113 aa protein, as do RHDV, RCV and Australian HaCVs (Figure 1). This sequence of subgenomic RNA is also conserved with respect to other lagoviruses starting at position 5,275. Potential cleavage sites described for the polyprotein processing of other HaCVs are conserved in HaCV_Bs12_1 (Droillard et al., 2018; Mahar et al., 2019) (Figure 1), and phylogenetic analysis of the entire ORF1 genome confirmed the results obtained with the *vp60* gene, with an HaCV branch supported by a bootstrap value of 100 (Figure 2a). The nucleotide identity and the amino acid identity comparisons of HaCV_Bs12_1 with hare caliciviruses (Australian and European) and a reference EBHSV

are shown on Figure 2b. In addition, in order to investigate a possible recombination event, a SimPlot analysis was performed that showed no apparent recombination with other known lagoviruses (data not shown).

3.2 | Screening of hares for lagovirus and prevalence estimation

All RT-PCR positive samples for HaCV were negative for EBHSV by RT-PCR employing specific primers (Velarde et al., 2016). Among the HaCV positive hares ($n = 57$) (Table 2), we were able to amplify and sequence the entire *vp60* gene from a total of 12 samples originating from Italy ($n = 9$), Austria ($n = 2$) and Germany ($n = 1$) (Figure 3, panels a–c). Based on the positive samples for HaCV detected in this study, we have estimated a country prevalence of 13.4% (IC95 11.26%–15.54%) in Italy, 10.4% (IC95 6.67%–14.13%) in Austria, 2.9% (IC95 0%–8.4%) in Germany and 0% in Spain. It is interesting to note that the hares sampled in Spain are only *L. granatensis*, from Navarre province (Figure 3, panel c), at the border with France. In Spain, *L. granatensis* is present throughout the peninsula, except in the northeast, where *L. europaeus* is exclusively found, and in the northwest, where *L. castroviejo* is restricted to a small mountainous area (Estonba et al., 2006).

3.3 | Sequence analysis of the vp60 gene

Sequencing of VP60 genes and blast analysis resulted in nucleotide identities among the 12 fully sequenced strains and HaCV_Bs12_1 (GB KR230102) ranging from 78% to 95.7%.

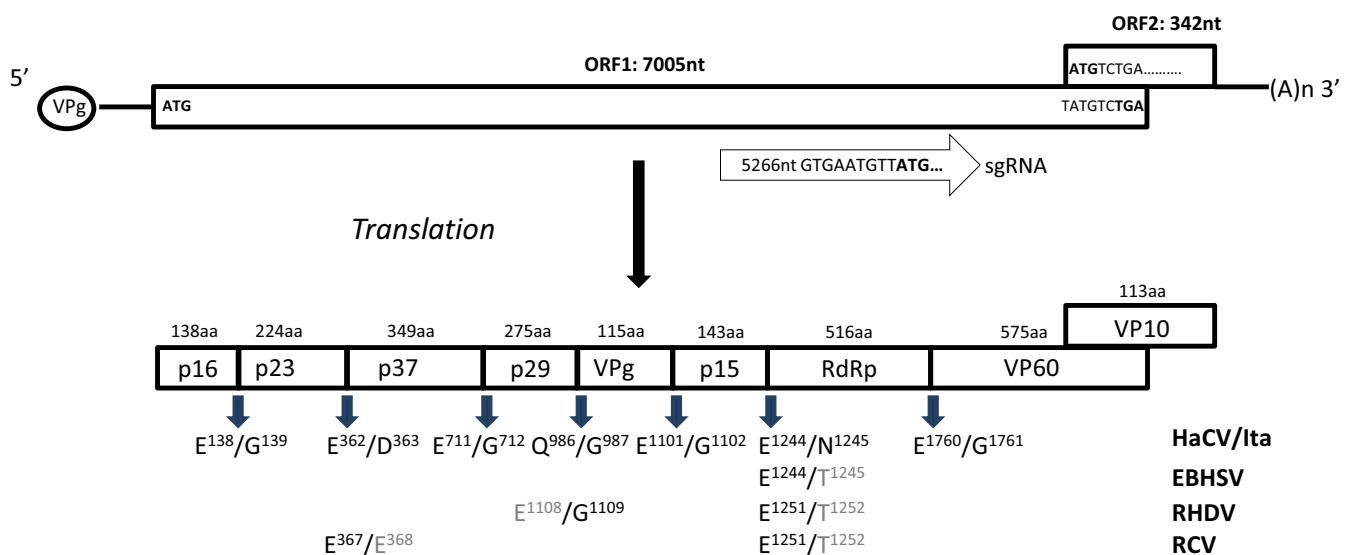


FIGURE 1 Schematic representation of the RNA genome organization of HaCV/Italy/Bs2012. The expected cleavage sites of the viral protease within ORF1, the name and the size (in amino acids, aa) of the cleavage products are indicated. Below the hypothetical polyprotein HaCV/Ita, are indicated in grey different cleavage sites reported for the other lagoviruses indicated on the right side

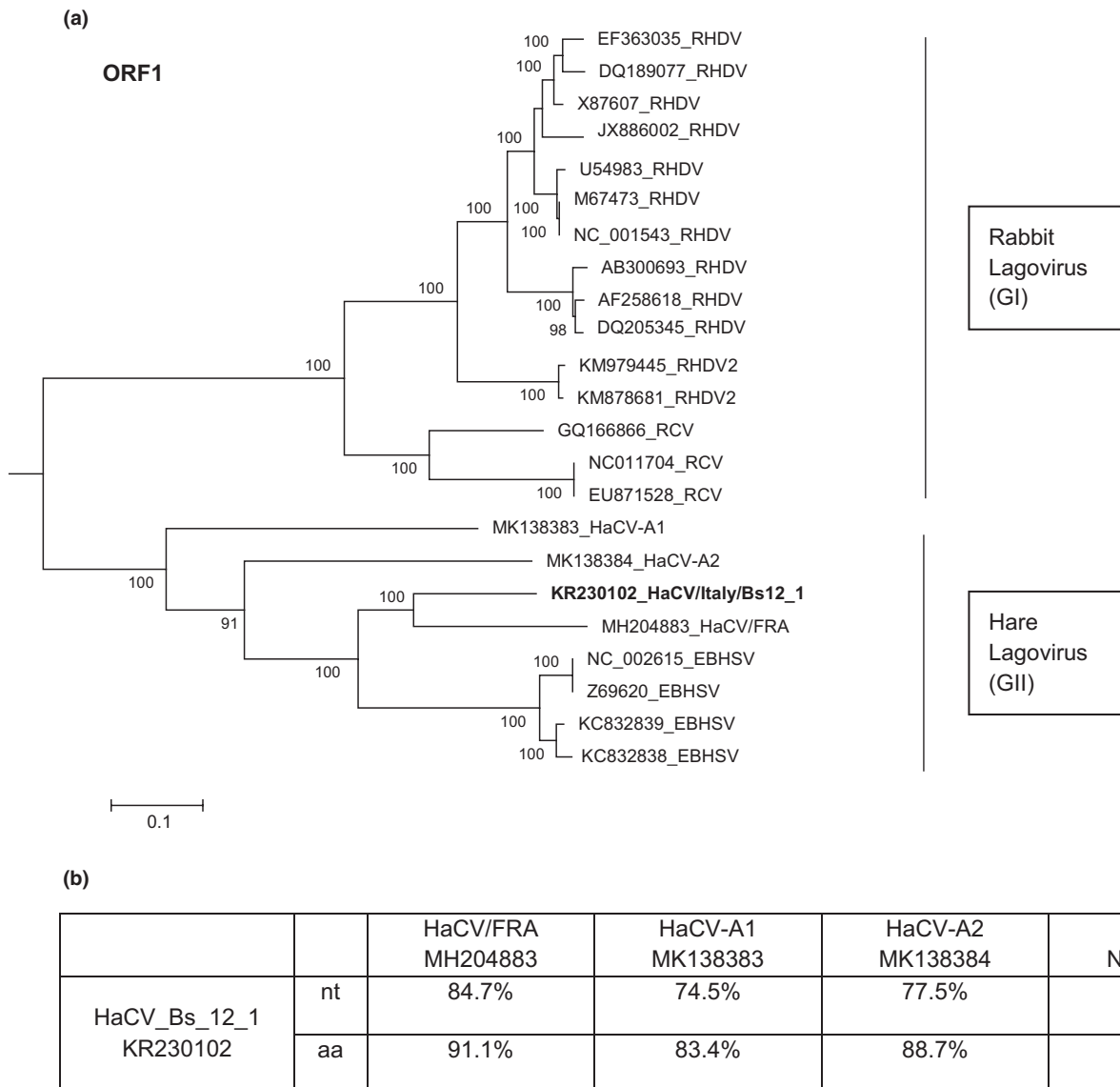


FIGURE 2 ORF1 genome analysis. (a) Maximum-likelihood phylogenetic tree with the nucleotide substitution model (GTR+G+I); the accession number of sequences obtained from GeneBank is shown in the taxa labels. The Italian sequence reported in this work is indicated in bold. (b) Full genome nucleotide identity and the amino acid identity comparisons of HaCV_Bs12_1, with HaCV/Fra (identified in France in 2015), two of three recently identified Australian HaCVs (A1 and A2) and a representative strain of EBHSV

The phylogenetic analysis performed only on the capsid sequences (Figure 4) supported evidence that all strains detected in this study clustered in a monophyletic HaCV group, including the first from Italy (2012) (HaCV_Bs12_1), as well as that detected in France (2015), and one of the three HaCVs found in Australia (2018) (HaCV-A2, MK138384). In contrast, the other two Australian strains (HaCV-A1, MK138383 and HaCV-A3, MK138385) and one French strain (MH992073_E15-226) cluster most closely with EBHSV by analysing the VP60 sequences only and, as suggested by Droillard et al. (2020), they belong to a new GII.3 genotype, whereas the Italian strains identified in this study belong to GII.2 genotype. The inability to amplify the full genome from all samples was likely due to viral RNA concentrations that were too low to permit successful complete amplification.

4 | DISCUSSION

After the first identification of non-pathogenic hare calcivirus (HaCV) in a hare farm located in the Brescia province (Northern Italy) (Cavadini et al., 2015), we conducted an epidemiological survey of wild hares to determine its geographic distribution and estimate its prevalence. The proximal tract of the duodenum was specifically sampled for this HaCV study because non-pathogenic RCVs have been predominantly detected in the small intestine of domestic and wild rabbits (Capucci et al., 1996; Strive et al., 2009).

We studied a total of 621 hares collected between 2010 and 2018 in Italy, and during 2011 in Germany, Austria and Spain. These animals were mostly collected from hunting activity and were therefore assumed to be healthy. We also examined hares found dead

TABLE 2 Total and RT-PCR-positive samples examined by country of origin and year of collection

Collection year	Italy		Germany		Austria		Spain		Total	
	Tot	Pos	Tot	Pos	Tot	Pos	Tot	Pos	Tot	Pos
2010	20	4	—	—	—	—	—	—	20	4
2011	33	1	104	3	106	11	89 ^c	0	332	15
2012	17	11	—	—	—	—	—	—	17	11
2014	198	11	—	—	—	—	—	—	198	11
2015	20, 1 ^a	1 ^a	—	—	—	—	—	—	20, 1 ^a	1 ^a
2016	1 ^b , 8 ^a	1 ^b , 1 ^a	—	—	—	—	—	—	1 ^b , 8 ^a	1 ^b , 1 ^a
2018	24 ^a	13 ^a	—	—	—	—	—	—	24 ^a	13 ^a
Total	322	43	104	3	106	11	89	0	621	57

^a*L. europaeus* var. *meridiei* from Pianosa Island.

^b*L. corsicanus* from Lucca Province.

^c*L. granatensis* from Navarre Province.

during passive surveillance. These animals did not have signs typical of Lagovirus diseases, and all laboratory diagnostic tests were negative for known lagoviruses. Molecular methods used for detecting HaCV viral RNA in hares included use of universal lagovirus RT-PCR primers and specific HaCV primers.

The high number of samples analysed showed that HaCV is circulating in brown hares in Italy, Germany and Austria. The average prevalence of HaCV in this study was 9.2% (IC 95 8.1%–10.3%), consistent with the recent identification of HaCV also in wild hares in France where the prevalence is around 10% (Droillard et al., 2020). Notably, according to Marchandau and Bertagnoli (personal communication), the estimated rate of wild rabbits infected with RCV in France is also similar (around 10%). Thus, considering that both viruses infect the intestinal tract and are highly genetically homologous, it could be hypothesized that the viral circulation and the detected prevalence may be similar.

The lack of detection of HaCV in Spain is not surprising. In fact, even if the serological surveys conducted in 2014 suggested that this agent could be present in the country (Velarde et al., 2016), those samples were originating from brown hares and not, like in this case, from Iberian hares. Indeed, the susceptibility of *L. granatensis* to lagovirus infections is still largely unclear (Lopes et al., 2014; OIE, 2016). In contrast, the HaCV prevalences in this study were lower than those found in Australia where three different HaCVs have recently been identified (Mahar et al., 2019). Such differences in the detection rate of HaCV could be related to ecological factors and population dynamics, since in Italy we noticed differences both from one year to the next, and according to the location (e.g. 45.4% on average in Pianosa Island). Similarly, in Australia, one HaCV strain was detected in a single hare, while the other two viruses were detected in 20% all animals tested and up to 30% at one site (Mahar et al., 2019), taking into consideration that the analysis was conducted in only two specific sites.

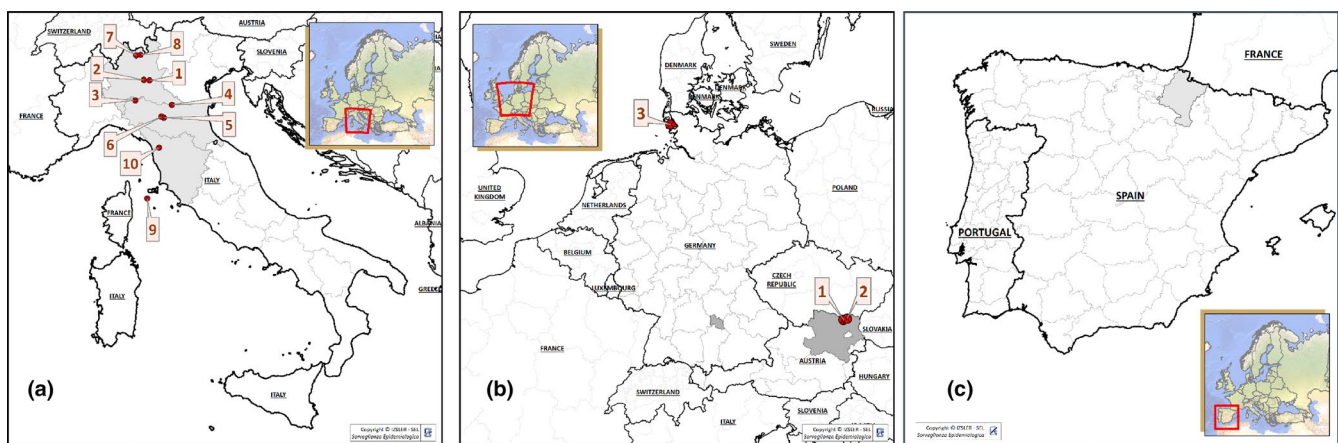


FIGURE 3 Maps showing locations of sampling. (a) Italy: in grey are highlighted Lombardy, Tuscany and Emilia Romagna regions. Numbers indicate positive HaCV samples sequenced: 1. HaCV/Italy/Bs2010, 2. HaCV/Italy/Bs2012, 3. HaCV/Italy/Pc2014, 4. HaCV/Italy/Mo2014, 5. HaCV/Italy/Re2014_1, 6. HaCV/Italy/Re2014_2, 7. HaCV/Italy/So2014_1, 8. HaCV/Italy/So2014_2, 9. HaCV/Italy/Lu2015, 10. HaCV/Italy/Lu2016. (b) Germany and Austria: in light grey are highlighted Großharras, Ungerndorf municipalities and Pellworm island. Numbers indicate positive HaCV samples sequenced: 1. HaCV/Austria/GH2011, 2. HaCV/Austria/UD2011, 3. HaCV/Germany/PE2011. (c) Spain: in grey is highlighted the Navarre region

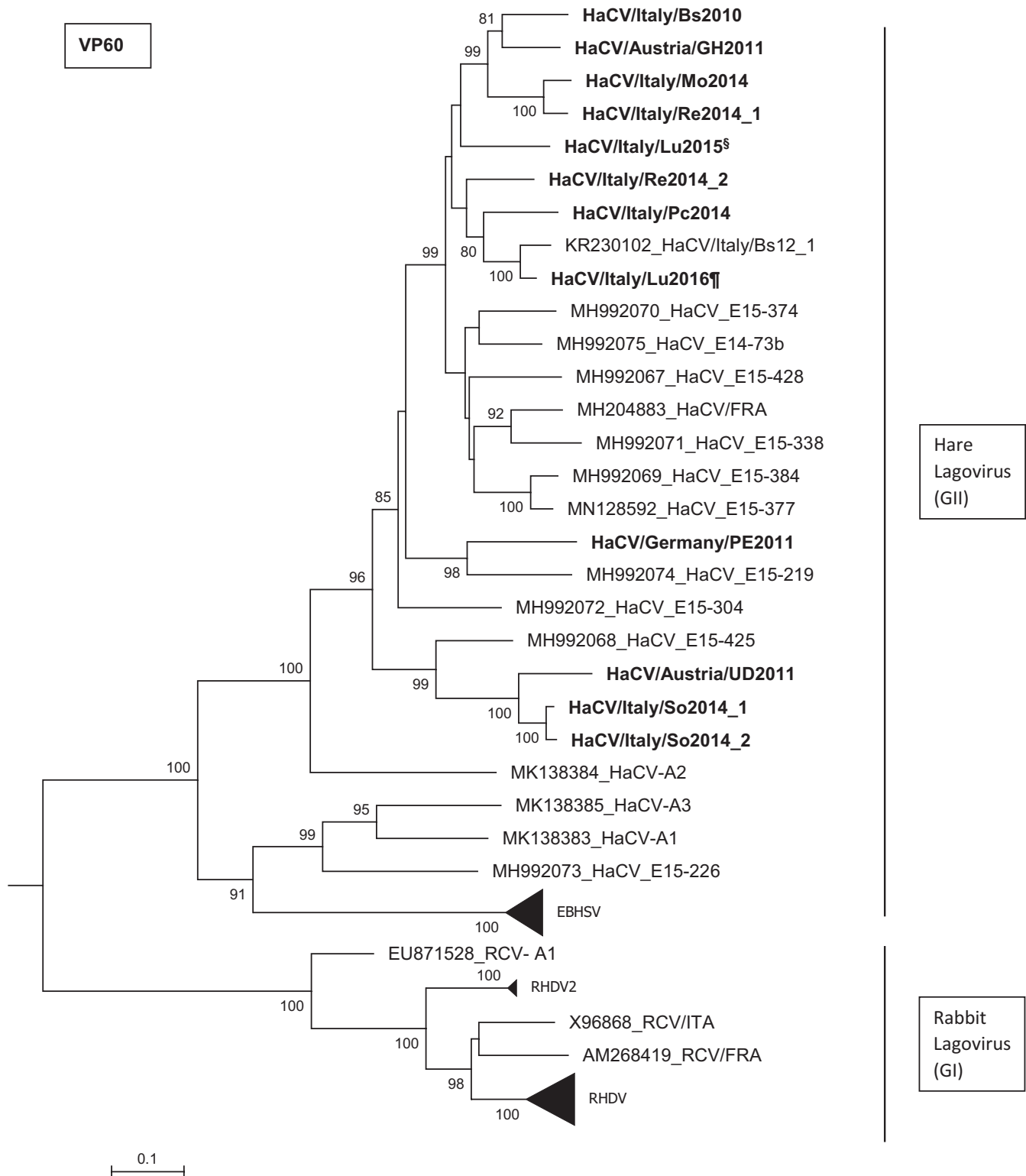


FIGURE 4 Maximum-likelihood phylogenetic tree of complete VP60 gene nucleotide sequences. The sequence panel included RHDV/RHDVa (GI.1a-d), non-pathogenic rabbit calicivirus: RCV-E1 (GI.3), RCV (GI.5) - X96868), RCV-A1 (GI.4), RHDV2 strains (GI.2), EBHSV strains (GII.1) and HaCV strains (GII.2) collected from an Italian breeding farm (KR230102), France (MH204883, MH992070, MH992075, MH992067, MH992071, MH992069, MN128592, MH992074, MH992072, MH992068, MH992073) and Australia (MK138383-138385). GenBank accession numbers for the HaCV strains identified in this study are as follows: KR349359, KR349360, KR349361, KR349362, MG781002, MG781003, MG781004, MG781005, MG781006, MG781007, MG781008 and MG781009. *SL. europaeus* var. *meridiei* from Pianosa Island; [¶]*L. corsicanus* from Lucca Province

Despite the relative low frequency of detection found in this study, our results are supported by the recent detection of HaCV strain from France (Droillard et al., 2018; Droillard et al., 2020), thus suggesting that this virus could be widespread in hare populations. Moreover, the detection of two distinct HaCVs in Australian hares and one in French hares, belonging to the same genotype GII.3 and sharing the same residue deletion in the VP60 sequence, indicates that lagoviruses were likely already present in brown hares at the time they were introduced into Australia from Europe in the 19th century, and thus, they have likely been circulating for longer than previously assumed. Because lagoviruses easily undergo complex evolutionary events, which are likely driven by multiple factors (environmental conditions, population genetics and densities, etc.), we cannot exclude that HaCV independently evolved in Australian hare populations, although we consider this to be far less likely. To better understand HaCV evolutionary history, a higher number of complete genomic sequences would be necessary.

It should be noted that the virological approach for determining the frequency and distribution of HaCV in hare populations alone is likely to largely underestimate the percentage of infected individuals. In fact, results of virological investigations are highly dependent on the presence of the etiological agent in its infectious, viremic or excretory phase, and therefore, they should be confirmed through the implementation of large-scale serum-epidemiological investigations. Moreover, considering that HaCV is non-pathogenic, the detection of specific antibodies produced after a natural infection, which, in analogy to other lagovirus infections, could last for a long time or even for a lifetime, could provide a better estimate of the real diffusion of the virus within hare populations. However, the difficulties we faced in carrying out a serum-epidemiological investigation included both the limited availability of hare sera, given most hares were shot during hunting, and the unavailability of ELISA tests specific for antibodies against HaCV with no cross-reactivity for antibodies against other lagoviruses (e.g. EBHSV and RHDV2). Indeed, a first preliminary study conducted with an ELISA test based on HaCV VLPs produced in the baculovirus system (Pezzoni et al., 2015) provided promising results. This test was carried out on a group of 18 sera from hares captured and sampled on the Island of Pianosa, and on a group of sera collected from the farm in which HaCV was identified for the first time, and, once validated, it could potentially be included in serum-epidemiological target surveillance plans in the future.

With regard to the presence of HaCV in Italy, it is of great interest that we found HaCV not only in *L. europeus* but also *L.e.meridiei* and in one *L. corsicanus*, thus indicating the capability of this virus to infect more hare species regardless of their genetic diversity. In particular, HaCV was repeatedly found in a population of a very ancient subspecies of brown hares, *L. europaeus* var. *meridiei* that inhabits Pianosa, a small island in the Tuscany Archipelago (Mengoni et al., 2018). Because Pianosa is a protected area, this hare species and the related microbiome/virome evolved as an isolated and

high-density population that was unaffected by translocation/restocking events associated with other species of hares. This again supports the hypothesis that HaCV have a long history of infecting hares.

In addition, since the same degenerated PCR primers used to detect HaCV in hares were also extensively used to search for non-pathogenic lagovirus in European rabbits in Australia and Europe, without identifying any HaCV-related sequence, we can suggest that HaCV, similarly to EBHSV, should be restricted to hare species and not present in rabbit populations.

In this study, we characterized the full genome sequence of the HaCV_Bs12_1 strain. This strain has the typical organization of lagoviruses, with ORF1 and ORF2 as the two major open reading frames (Clarke & Lambden, 1997; Le Gall, Huguet, Vende, Vautherot, & Rasschaert, 1996; Wirblich et al., 1994). In comparison with the available full sequences of EBHSV and HaCVs, this strain has one deletion in amino acid position sixty-five of VP2. This deletion probably does not interfere with maturation and assembly of HaCV particles, based on the comparative observation that feline calicivirus (FCV) has functional domains localized at the N and C terminus of VP2 (Sosnovtsev, Belliot, Chang, Onwujiwe, & Green, 2005).

Our work identified 12 HaCV capsid sequences that appear to form a monophyletic group of several cocirculating European strains. The detected nucleotide diversity (around 18%) did not appear to be related to the year or location of sample collection. Thus, the lack of more extensive sampling should be the likely cause of this genetic diversity hitherto highlighted (Droillard et al., 2020; Mahar et al., 2019 and our observations).

Note that in Australia, three HaCVs (A1, A2 and A3) have been described: two of them (A1 and A3), considering the vp60 gene, are more similar to EBHSV and divergent to European HaCVs (as also recently described by Droillard et al., 2020), while the third one (A2) is more similar to the European HaCV strains identified to date. Such higher similarity of the two non-pathogenic lagoviruses A1 and A3 to EBHSV suggests a more recent common ancestor between them (Mahar et al., 2019).

As mentioned above, such diversity among different HaCV strains could be due to an independent genetic evolution resulting from recombinant events that are selected by external factors, such as the distribution of local populations in the wild. Therefore, it is likely that some of these HaCV strains represent also a new genotype (Droillard et al., 2020; Le Pendu et al., 2017; Mahar et al., 2019), although the lack of further sequences does not currently allow a correct classification.

The natural circulation of non-pathogenic viruses in domestic and wild hare populations illustrates the importance of determining the impact that these viruses might have on the distribution and transmission of pathogenic viruses in different hare species. In particular, the potential to induce cross-protective antibodies or limit infection by related strains, including virulent viruses (EBHSV but also potentially RHDV2) should be determined.

To better understand the role that HaCV plays in the epidemiology of lagovirus infections, it will be important to conduct

extensive serum-epidemiological investigations. However, diagnostic methods with sufficient specificity need first to be developed and made available before such analyses can be performed. In fact, given the sharing of many preserved common epitopes in all the viruses of the genus *Lagovirus* identified to date, both pathogenic and non-pathogenic, either in the rabbit or in the various hare species, it will be crucial to develop and validate tests, preferably based on monoclonal antibodies, capable of selectively binding HaCV surface epitopes, which are more often endowed with neutralizing activity.

In conclusion, this study confirms the need to further understand the biology and characteristics of the genus *Lagovirus*. There is a need to sample and examine not only dead animals of different species of domestic and wild lagomorphs, but also healthy subjects, with molecular and metagenomic analyses, to fill the gaps still existing in phylogeny of these viruses and their evolutionary mechanisms.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as samples were collected from already deceased animals. The sample collection was performed using procedures in compliance with international guidelines.

CONFLICT OF INTEREST

The authors declare no financial or personal relationships with other people or organizations that could inappropriately influence their work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank® at <https://www.ncbi.nlm.nih.gov/genbank/>, reference numbers: KR349359, KR349360, KR349361, KR349362, MG781002, MG781003, MG781004, MG781005, MG781006, MG781007, MG781008 and MG781009.

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