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Potential role of wolf (*Canis lupus*) as passive carrier of European brown hare syndrome virus (EBHSV)

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ABSTRACT

European brown hare syndrome virus (EBHSV) was detected in a faecal swab collected from a wolf carcass in Northern Italy. The full-length genome of the EBHSV WOLF/17/2016/ITA strain was determined. In the VP60 capsid gene, the wolf strain displayed the highest genetic identity (99.2–99.1% nucleotide and 99.6–99.7% amino acid) with two EBHSV strains recently found in the intestinal content of a red fox and in the spleen and liver of a hare in Northern Italy. This finding poses interrogatives on the potential role of carnivores as EBHSV passive carriers, favoring the introduction and spread of the virus among different hare populations.

European brown hare syndrome virus (EBHSV) is the cause of a highly contagious disease associated with severe necrotizing hepatitis affecting European hares (*Lepus europaeus*) and, to a lesser extent, other hare species such as *Lepus timidus* and *Lepus corsicanus* (Frölich and Lavazza, 2008). The eastern cottontail (*Sylvilagus floridanus*) also seems susceptible to EBHSV infection (Lavazza et al., 2015).

European brown hare syndrome (EBHS) was originally described in Sweden in 1980 (Gavier-Widén and Mörner, 1991). It was identified as a viral disease in 1989 (Gustaffson et al., 1989; Lavazza and Vecchi, 1989) and since then, the disease has been observed in several European countries (Billinis et al., 2005; Chasey and Duff, 1990; Duff and Gavier-Widén, 2012; Frölich and Lavazza, 2008; Lopes et al., 2014) including Italy, where is considered endemic since its first description in the late 1980s (Capucci et al., 1991; Scicluna et al., 1994).

EBHSV is a small non-enveloped virus of approximately 30 to 35 nm in diameter classified together with the rabbit haemorrhagic disease virus (RHDV) in the *Lagovirus* genus, within the *Caliciviridae* family (King et al., 2012). The EBHSV icosahedral capsid surrounds an approximately 7.4 kb positive-sense single-stranded RNA genome organized in two open reading frames (ORFs). ORF1 encodes a polyprotein of ~2334 amino acids (aa) that undergoes protease processing to produce several nonstructural proteins, including a RNA-dependent RNA polymerase (RdRp) and a capsid protein (VP60). ORF2 encodes a small protein (VP10) with an unknown function (Le Gall et al., 1996). EBHSV is easily transmitted by direct contact through the oro-faecal and aerosol routes but, giving the high resistance of virions in the environment, indirect transmission can often occur by contaminated equipment, vehicles and tools as well as humans, insects, birds, and rodents (Frölich and Lavazza, 2008). Also, it has been hypothesized that generalist predators and particularly carnivores, that have consumed EBHSV-infected hares, even if they do not develop infections or clinical symptoms (Frölich et al., 1998), can spread the virus through faecal droppings in the environment. It has been shown for EBHSV by Chiari et al. (2016) that found EBHSV in the gut content of a fox, and it has been also experimentally demonstrated by Simón et al. (1994), who detected the EBHS-related virulent lagovirus, agent of rabbit haemorrhagic disease (RHD), in faecal contents of dogs experimentally fed with homogenized liver from rabbits that died of RHD.

Herewith, we report the identification and the full-genome characterization of a EBHSV strain detected in a faecal specimen collected from a wolf found dead in North-Western Italy.

Between February 2015 and April 2017, rectal swabs were collected from 41 wolves found dead and submitted for necropsy to the National Reference Center for Wild Animal Diseases (CeRMAS) and Torino

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University (Italy). All the samples were screened for common canine viral pathogens by using either conventional or quantitative PCR and RT–PCR. Calicivirus RNA was identified in a faecal swab, collected from a wolf found dead in the province of Alessandria (lat. 44.504; long. 8.3442) in Piemonte Region, by using a broadly reactive primer pair, p289-p290, targeted to highly conserved motives of the polymerase complex (Jiang et al., 1999). The same specimen was negative when re-screened with primers specific for noroviruses (Vennema et al., 2002), vesiviruses (Martella et al., 2015), sapoviruses (Bodnar et al., 2016) and feline calicivirus (Marsilio et al., 2005). Upon sequence analysis of the 315-bp fragment of the RdRp region using BLAST (www.ncbi.nlm.nih.gov/blast) and FASTA (www.ebi.ac.uk/fasta33), the highest genetic relationship was found with EBHSV (92.0–93.0% nucleotide [nt] identity). The sample tested positive when using EBHSV specific primers EBHS-148F and EBHS-578R (Velarde et al., 2016).

To determine the sequence and genome organization of this putative virus (EBHSV/WOLF/17/2016/ITA), a 3.4-kb region at the 3′ end of the genome was amplified by a 3′ RACE protocol. The cDNA was synthesized by SuperScript III First-Strand cDNA synthesis kit (Invitrogen Ltd., Italy) with an oligo(dT). The PCR was performed with TaKaRa La Taq polymerase (Takara Bio Europe, France) using consensus primers. The complete 3.4 kb sequence was determined by a primer walking strategy (Table 1). To obtain the 5′ end sequence of the genome, a 5′ RACE kit was used (Invitrogen Ltd., Italy). Sequence editing and multiple alignments were carried out with the Geneious software package version 9.1.6 (Biomatters Ltd., New Zealand). Phylogenetic tree was inferred by using the MEGA v3.0 (Kumar et al., 2004). The full-length genome of the EBHSV WOLF/17/2016/ITA was deposited in GenBank under accession number MF356366. Two open reading frames (ORFs), of 7005 nt (ORF1) and 345 nt (ORF2) in length, were mapped. ORF1 encodes a putative large polyprotein of 2334 aa in length, containing the replicative proteins and, at the 3′ end, the 1731 nt-long (576 aa) VP60 capsid region. ORF2 encodes a putative 114-aa long protein (VP10). By sequence comparison, the highest identities (91.0–93.0% nt and 96.0–97.0 aa) were to the three full-genomes of EBHSV, namely strain GD/FR/1989 (Z69620) (Le Gall et al., 1996), 04022–10/Sw/1982 (KC832838) and 04021-9/Sw/1982 (KC832839) (Lopes et al., 2013), followed by RHDVs (71.0–72.0% nt and 78.0–79% aa). Since only three complete genomes of EHBSV are currently available in the databases, our comparative analysis was limited to the VP60 capsid coding region (1731 nt) of ORF1. Strain EBHSV/WOLF/17/2016/ITA displayed the highest identity (99.2% nt and 99.7% aa) to the strain Fox/Bs-15/ITA (KU961677) recently detected in the intestinal content of a red fox shot in Lombardia region (Italy) in 2015 and to the strain Hare/Bs-15/ITA (KU961678) (99.1% nt and 99.7% aa) identified in the liver and spleen of a hare found dead in the same geographic area and period (Chiari et al., 2016). Identity to other EBHSVs available on the databases was 90.0–95.0% nt and 94.0–99.0% aa. Based on sequence and phylogenetic analysis of the full-length VP60 capsid gene (Fig. 1), the strain WOLF/17/2016/ITA segregated along with the EBHSVs Fox/Bs-15/ITA and Hare/Bs-15/ITA (bootstrap value 100%) detected in Italy in 2015 (Chiari et al., 2016) within a cluster containing French and Swedish EBHSV strains (Lopes et al., 2014) collected from 2001 to 2008 (bootstrap value 81%). On the whole, these results suggest the spread of EBHSV strains highly genetically related to each other (99.6–99.7% aa identity), within a short time span, in hares and their predators, wild canids, in contiguous geographical areas of North Italy.

Table 1
List of primers used in this study. Nucleotide position refers to the sequence of the prototype strain EBHSV-GD/FR (GenBank accession no. Z69620).

Oligonucleotide	Position	Sequence (5'to 3′)	Sense
5′ UTR	9–29	ATGGCGGTTGCGTCGCGCCCT	+
789	803–824	GCTTAAAACCTTGGCCGCAATT	+
885	885–903	CGAGCAAATTCACAGGTTT	–
1081	1081–1100	TTGACAAGTTTGAGGACTCA	+
1269	1275–1294	TCCTTGGCAACCATATGCTG	–
1800	1773–1795	GACAAGGTTGAAAACAAAAACAA	+
1922	1926–1947	ACCCATGTCTGGTTGACTGCCA	–
2529	2530–2551	TGAAGTTGATTTGGGCATTGCG	+
2898	2898–2915	AACAAGGTGGTTGTTGCA	–
3000	2988–3007	ATCCTGGCACCGCGCCCGCG	–
3172	3182–3201	GGACGAGGCCCACGTGACAG	+
4029	4049–4071	GAAGGGTGAACGACAAATGAACC	+
4029	4049–4071	GGTTCATTTGTCGTTCACCCTTC	–
4546	4546–4568	TTGTTCACAGCAGTCAGCAAGGA	–
4708	4709–4730	GCTTTGGTCTGCAGCAGTTTAT	+
5125	5125–5145	CTCATGGTGAGGAGTTCTTCG	+
5269	5269–5288	CTCCATAACATTCACAAAA	–
5518	5518–5539	AGAATGCTACCGGGTGCATCGG	–
6048	6048–6069	CCAATTGTCGGGCTCCAGCCCG	+
6762	6762–6783	CTGTCCCTGACAAACTACTCCT	+
7020	7020–7039	AGGACTAACATTGGCTGGAG	–
7289	7289–7310	CTATTATTAAAACTTTTAATCT	–
7430	7422–7440	AATTTGCTTTAAACTATAA	–

In our analysis, we wanted to investigate whether the virus could pass the mucosal barrier thus localizing and replicating in extra-intestinal sites or if its presence was restricted to the faecal contents as consequence of the intestinal transit of remains of an infected prey. Thus, we analyzed the liver and the spleen of the wolf by RT-PCR using caliciviruses generic primers p289-p290 (Jiang et al., 1999) and EBHSV specific primers (Velarde et al., 2016), but both the assays resulted negative. In addition, hare and wolf DNAs were identified in the faecal sample in PCR using a pair of primers able to detect hare DNA (Fw: 5′ - TAATCAACATTAGACCATTAC - 3′ and Rev.: 5′ - GTCTGGATGTTGAT TAAGG - 3′) (Chiari et al., 2016) and a specific primer set for *Canis lupus* DNA (Fw: 5′ - CTTTCTGGCATTCATTCTTTC – 3′ and Rev.: 5′ - TGATTA GCAAACCATATGTTG – 3′), confirming the hypothesis that the wolf enteric tract contained the remains of a EBHSV-infected hare. To date, there is no evidence that EBHSV may infect or cause disease in carnivores. However, even if virological studies on RHDV have ruled out the possibility to reproduce the disease in dogs (Simón et al., 1994), the presence of specific anti-RHDV antibodies in foxes suggests that abortive or subclinical infections might occur (Frölich et al., 1998; Philbey et al., 2005). Yet, it is difficult to speculate whether wolves are susceptible or not to EBHSV infection, and only large serological surveys or experimental infections could firmly address this hypothesis. EBHSV RNA has been already detected in the intestinal content of a carnivore, i.e. in a free-ranging red fox from North Italy (Chiari et al., 2016), but virological investigations ruled out the presence of EBHSV in the liver, spleen and mesenteric lymph nodes. Also, IgG specific antibodies for EBHSV were not identified in the fox. Interestingly, in the gastro-intestinal content of the EBHSV-positive fox, hare DNA was detected, suggesting that the presence of the EBHSV was due to the predation of infected hares. Likewise, we found hare DNA in the wolf intestinal content, making it a reliable hypothesis. This poses interrogatives on the potential role of carnivores as EBHSV mechanical, passive carriers, favoring the introduction and spread of EBHS among different hare populations. Also, upon genome characterization, it was clear that EBHSV strains similar to virus 17/2016/ITA, are circulating in North-Italy in the same time span. Indeed, the EBHSV-positive faecal specimen was collected in a geographical area (Alessandria, Piemonte Region) with a high density of brown hares and cottontails, and where both wolves and foxes have been reported. Experimental infections of seronegative hares by administration of faecal suspensions of EBHSV-positive carnivores could be useful to assess if this virus is still viable and infectious for hares and if carnivores may act, at least, as passive carriers of the virus.

A limit of this study was the fact that only three complete genomes of EBHSV are available in the databases (interrogation on October 2017). Accordingly, it was not possible to look for possible genetic hallmarks in the genome of the EBHSV strain detected in the wolf carcass. Sequencing of additional EBHSV strains could help to