Identification and characterization of a novel tick-borne flavivirus subtype in goats (Capra hircus) in Spain

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In 2011, a neurological disease was reported in a herd of goats (Capra hircus) in Asturias, Spain. Initial sequencing identified the causative agent as louping ill virus (LIV). Subsequently, with the application of whole genome sequencing and phylogenetic analysis, empirical data demonstrates that the LIV-like virus detected is significantly divergent from LIV and Spanish sheep encephalitis virus (SSEV). This virus encoded an amino acid sequence motif at the site of a previously identified marker for differentiating tick-borne flaviviruses that was shared with a virus previously isolated in Ireland in 1968. The significance of these observations reflects the diversity of tick-borne flaviviruses in Europe. These data also contribute to our knowledge of the evolution of tick-borne flaviviruses and could reflect the movement of viruses throughout Europe. Based on these observations, the proposed name for this virus is Spanish goat encephalitis virus (SGEV), to distinguish it from SSEV.

Louping ill virus (LIV) is endemic in upland areas of the UK and Ireland and causes a febrile illness in sheep, cattle, grouse and some other species that can progress to fatal encephalitis (Jeaffres et al., 2014). The occurrence LIV cases is closely associated with the presence of its arthropod vector, the Ixodes ricinus tick, with a geographical bias towards upland grazing areas of the UK (Jeaffres et al., 2014). LIV is a positive-sense RNA virus belonging to the Flavivirus genus. Molecular phylogeny based on the gene encoding the envelope protein suggests that there are four geographically separate lineages of LIV within the British Isles: genotype 1 (Scotland and England), genotype 2 (Scotland), genotype 3 (Wales) and genotype 4 (Ireland) (Gao et al., 1997). Reports of LIV outside the British Isles are rare. A LIV isolate reported from Norway originated from the UK, grouping with the genotype 1 isolates from Scotland & England (Gao et al., 1997a, 1997). However, LIV also demonstrates a high degree of genetic homology to tick-borne encephalitis virus (TBEV) and other mammalian tick-borne viruses, particularly Spanish sheep encephalitis virus (SSEV). Other related viruses include Greek goat encephalitis virus (GGEV) and Turkish sheep encephalitis virus (TSEV) (González et al., 1987; Gao et al., 1993b; Marin et al., 1995; Gritsun et al., 2003; Grard et al., 2007). The current taxonomic classification, as specified by the International Committee on Taxonomy of Viruses,
states that these viruses are all subtypes (subspecies/variants) within the LIV species of mammalian tick-borne flaviviruses, with the exception of TBEV, which constitutes a separate species (Pletnev et al., 2011).

SSEV was detected in the Basque region of northern Spain during the 1980s, causing clinical disease in sheep identical to louping ill infection, with indistinguishable histological changes in the brain and neutralizing antibodies that cross-reacted with LIV (González et al., 1987). In September 2011, severe disease and mortality affected a recently purchased herd of Berrymea goats in the Asturias region of northern Spain (Balseiro et al., 2012). Clinical disease was detected 4 weeks after purchase, with one animal exhibiting hind leg lameness. This rapidly progressed to incoordination, fevers, tremors and bulging eyes, with death after 2 days. Subsequently, 17 goats within the same herd exhibited similar clinical signs and died over a four-month period. The herd was vaccinated during January 2012 with a single dose of louping ill vaccine (inactivated LIV vaccine; Intervet), although this did not prevent the death of three more goats, the latest in June 2012. Initial histopathological examination identified severe non-suppurative encephalitis in a range of brain sections from three of the affected goats, leading to the initial clinical diagnosis of SSEV. Subsequently, molecular analysis of brain tissue identified the virus as LIV, as sequence for the gene encoding the envelope protein was 94% identical to a LIV strain from the UK and 93% identical to SSEV (Balseiro et al., 2012). Despite the close proximity of the Basque country to Asturias (400 km), primary molecular analysis showed that the virus involved in the present study was not the same as that identified previously in the Basque country, suggesting an independent origin. However, this initial molecular analysis was based upon a short 231 bp amplicon and therefore further genomic characterization of the Spanish goat viral isolate was required. In this study, the complete genome of the Spanish goat viral isolate has been fully sequenced and phylogenetic analysis has been undertaken.

The Spanish goat isolate (APHA reference Arb408; original reference AstGoatIREC2011) was originally isolated from the brain of a goat in the Asturias region of Spain in 2011 and subsequently passaged in ISE6 Ixodes scapularis tick cells. RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen), with reverse transcribed using the Hot Start DNA polymerase (Novagen) according to the manufacturer’s instructions. Amplicons were purified using the QIAquick PCR Purification kit (Qiagen), and sequenced using ABI Big Dye kit. The 3’ and 5’ UTRs were sequenced using the 3’ and 5’ RACE systems (Invitrogen), respectively, along with Platinum Taq Polymerase High Fidelity (Invitrogen). Details of all primers used for genomic sequencing are available upon request. An archival LIV (APHA reference Arb126; original reference LI 3/1) isolated from an infected sheep from Oban, Scotland, in 1962 also underwent genome sequencing, in order to provide additional sequence data for phylogenetic comparison. The genome sequences of the Spanish goat isolate (Arb408) and the UK LIV isolate (Arb126) were deposited in GenBank under accession numbers KP144332 and KP144331, respectively. Sequences were analysed and edited using Editsq, Seqman and MEGALIGN within the DNASTAR Lasergene 8 software package. The 10 245 bp ORF nucleotide sequences were compared to additional tick-borne flaviviruses (detailed in Table S1, available in the online Supplementary Material), using maximum-likelihood phylogenetic trees reconstructed within the MEGA5 package (Hasegawa–Kishino–Yano substitution model), with bootstrap values corresponding to 1000 replications. An additional maximum-likelihood tree was also reconstructed within the MEGA5 package to compare the envelope gene (E-gene) sequences from a larger number of isolates (detailed in Table S1).

Whole genome comparison demonstrated that the Spanish goat isolate (Arb408) shared 89.4% homology with SSEV and between 88.7–89.3% homology with the three LIV isolates included in the phylogenetic analysis. Phylogenetic analysis of 10 245 bp of nucleotide sequence encompassing the polyprotein coding region demonstrated that Arb408 grouped within a clade, which encompassed all of the LIV isolates along with SSEV (Fig. 1, Table S1). Within this clade, Arb408 grouped more closely with SSEV than with LIV, with good bootstrap support (100%) and it is possible that Arb408 constitutes a new variant of SSEV. However, the extended branch length suggested that SSEV and Arb408 are still significantly divergent and that Arb408 may constitute a new subtype (subspecies) within the LIV species of tick-borne flaviviruses. Additional phylogenetic comparison of the E-gene for a larger number of tick-borne flaviviruses demonstrated a similar pattern, with Arb408 and SSEV grouping with the LIV isolates (Fig. 2, Table S1). However, both SSEV and Arb408 have extended branch lengths. For both datasets, similar groupings were observed with phylogenetic trees created using the neighbour-joining method (data not shown). Through further analysis of the envelope protein amino acid sequence, a novel motif was identified within the envelope protein of Arb408 between 232–234 aa, at a site previously identified as a tri-peptide genetic marker for distinct flavivirus species (Marin et al., 1995) with biological importance for virulence (Shiu et al., 1991). The study by Marin et al. (1995) identified unique tri-peptide motifs for a number of different tick-borne flaviviruses, including LIV and SSEV, which were asparagine–proline–histidine (NPH) and alanine–glutamine–arginine (AQR), respectively (Marin et al., 1995). In this study, the motif for Arb126 (LIV) was confirmed as NPH, as for the majority of the other LIV isolates analysed. Furthermore, within the LIV/SSEV clade, SSEV contained the motif AQR (as previously described) and we have identified the unique motif for Arb408 as glycine–proline–arginine (GPR) (Fig. 2). This observation, along with the branch distances in phylogenetic analyses,
suggests that the Spanish goat isolate, Arb408, may be proposed as a new subtype (subspecies) within the LIV species of the mammalian tick-borne flavivirus group; tentatively named Spanish goat encephalitis virus (SGEV). The older lineages, GGEV and TSEV, share the same tri-peptide motif alanine–leucine–glycine (ALG), which appears to have mutated to alanine–glutamine–asparagine (AQN) with the divergence of a separate virus species, European TBEV. Subsequently, the divergence of SSEV led to further mutation and a tri-peptide motif of AQR. Divergence of SGEV followed, with the tri-peptide mutating to GPR, a motif shared by the Irish lineage of LIV suggestive of a direct introduction of a common precursor virus into Ireland from Spain. In addition, the Scottish, English, Welsh and Norwegian LIV isolates evolved the tri-peptide NPH (or asparagine–proline–tyrosine; NPY). Despite the potential for variation in the evolution rates for different genes within the genome, the trees reconstructed in this study demonstrated a similar evolutionary pattern to previously published trees using the NS3 and NS5 genes (Grard et al., 2007). However, there is the potential for evolutionary rates to be hidden by strong purifying selection since nucleotide substitution models cannot take into account variability in selection pressures (Wertheim & Kosakovsky Pond, 2011). This fact may lead to underestimation of the origin of viral clades, although it is more likely to influence long internal branches of a tree rather than short recent branches as shown for LIV and SSEV in this study.

The complete genome of SGEV was 10,870 bp in length, slightly shorter than that of the LIV isolate (Arb126) at 10,880 bp. The difference in length was due to variation in the length of the 3′ UTR, with the 5′ UTR and all coding genes being of identical length. A comparison of individual gene size and translated amino acid size for both isolates is summarized in Table 1. The variation observed in genome length was the result of a 10 nt deletion in the variable region of the 3′ UTR of SGEV. Studies with the related flavivirus TBEV have shown that the length of the variable region has no effect on either RNA replication or efficiency of translation in mammalian cells (Hoenninger et al., 2008). In comparison, the core region of the 3′ UTR contains RNA secondary structures essential for replication, where even small deletions can significantly increase lethality in mice (Hahn et al., 1987; Wallner et al., 1995; Mandl et al., 1998). However, the core region remained largely conserved for both LIV and SGEV, possibly reflected by the similarities in clinical disease observed for each virus. Although severe encephalitis has been previously described in goat kids experimentally and naturally infected with LIV (Papadopoulos et al., 1971; Reid et al., 1984), it has not been reported previously in adult goats. This suggests possible differences in pathogenesis and pathology of SGEV in this species (Balseiro et al., 2012). Histopathological examination for the presence of LIV in the brain of a naturally infected adult goat that died in Scotland, showed only chronic low grade lesions (Gray et al., 1988) and the brains of experimentally infected adult goats showed mild non-suppurative meningoencephalitis, although the lesions were more severe in one goat that developed clinical signs (Reid et al., 1984). However, the reasons for this difference are unclear.
Additionally, the amino acid sequence for the envelope protein was relatively conserved, whilst the highest substitution rate for translated amino acid sequences were observed for the C, NS2a and NS4b proteins. This suggests that there may be functional differences between these proteins for SGEV and LIV, although this assumption would have to be confirmed through reverse genetics-based studies. The C, NS2a and NS4b proteins all have essential roles during virus replication (Mackenzie et al., 1998; Kofler et al., 2002; Liu et al., 2003; Umareddy et al., 2006; Leung et al., 2008), and the NS2a and NS4b proteins have also been shown to block the host antiviral response via inhibition of the type 1 IFN response (Liu et al., 2005; Muñoz-Jordán et al., 2005; Tu et al., 2012).

Genetic characterization of SGEV indicates that it is distinct from SSEV despite sharing virulence for small ruminants. The evolution of these tick-borne flaviviruses may have resulted from the intensification of sheep and goat farming throughout Europe, and human migration, which was associated with movement of livestock and the exoparasites associated with them, which may have led to subsequent diversification. Therefore, it is possible that changes in land use, along with the movement of large populations of humans and associated domestic animals, may have led to fragmentation of tick habitat and may have contributed to the evolution of tick-borne viruses with virulence for sheep, goats and humans. The emergence of SSEV in Spain may reflect the movement of peoples and their livestock from Turkey and the Middle East into Moorish Spain. Evolutionary molecular studies investigating the origins of LIV have suggested that emergence in Ireland occurred less than 800 years ago and subsequent

**Fig. 2.** Phylogenetic analysis of 1488 bp of E-gene coding sequence. Maximum-likelihood tree reconstructed using MEGA 5, with bootstrap values corresponding to 1000 replicates, and OHFV-Kubrin as the outgroup. Only bootstrap support values greater than 70% are shown. Tri-peptide motifs between 232–234 aa are shown (three letter amino acid code is in italics). Isolates highlighted blue (LIV), purple (European TBEV), green (Siberian TBEV), pink (Far-eastern TBEV) and red (SGEV); all other isolates highlighted black.
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References


Table 1. Size of genes encoding viral proteins and UTR from LIV (Arb126) and the Spanish goat isolate (Arb408)

<table>
<thead>
<tr>
<th>Genome region</th>
<th>Gene size (bp)</th>
<th>Encoded protein size (aa)</th>
<th>Amino acid substitutions</th>
</tr>
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<tbody>
<tr>
<td>LIV (Arb126)</td>
<td>Spanish goat (Arb408)</td>
<td>Total</td>
<td>%</td>
</tr>
<tr>
<td>5' UTR</td>
<td>132</td>
<td>132</td>
<td>NA</td>
</tr>
<tr>
<td>C</td>
<td>336</td>
<td>336</td>
<td>112</td>
</tr>
<tr>
<td>prM</td>
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<td>279</td>
<td>93</td>
</tr>
<tr>
<td>M</td>
<td>225</td>
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</tr>
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<td>NS2b</td>
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<tr>
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</tr>
<tr>
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<td>149</td>
</tr>
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<tr>
<td>NS5</td>
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<td>2709</td>
<td>903</td>
</tr>
<tr>
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<tr>
<td>Total length</td>
<td>10 880</td>
<td>10 870</td>
<td>–</td>
</tr>
</tbody>
</table>

NA, Not applicable.

divergence between viruses during the past 300 years; this appears closely associated with the movement of livestock within the British Isles (McGuire et al., 1998). In Europe, the past 300 years have been associated with extensive economic growth, fuelled by the agricultural and industrial revolutions. Dramatic changes in farming practices may have influenced the evolution of viruses with specific tropism for sheep and goats. Similarly, changes in land use, farming practices and tick habitats in different regions may have contributed to the evolution of LIV and the sheep/goat flaviviruses (Marin et al., 1995). The divergence of the Irish and Welsh LIV isolates appeared to occur prior to that of the English and Scottish isolates, suggesting that LIV entered the UK from mainland Europe via Ireland, before entering Wales and then England and Scotland (McGuire et al., 1998). The route of introduction may never be determined with certainty, but could be associated with movement of infected animals, importation of infected ticks on livestock or potentially by infected ticks on migratory birds. In conclusion, the Spanish goat isolate constitutes a tentative new subtype (subspecies) within the loaping ill species of tick-borne flaviviruses and may be tentatively designated SGEV.


