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NEW VARIANTS OF EUROPEAN BROWN HARE SYNDROME VIRUS STRAINS IN FREE-RANGING EUROPEAN BROWN HARES (*LEPUS EUROPAEUS*) FROM SLOVAKIA

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ABSTRACT: Investigations regarding European brown hare syndrome virus (EBHSV) in European brown hares (*Lepus europaeus*) in Slovakia were undertaken in order to detect the possible presence of EBHSV and to evaluate its phylogenetic position. Liver and/or serum samples were obtained from 135 European brown hares shot by hunters in eight regional hunting areas. From 36 animals corresponding liver and serum samples were available; from the remaining 49 and 50 animals only liver or serum samples were examined, respectively. Samples were tested for antibodies against EBHSV and for viral RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) and RT-PCR products were subsequently sequenced. Additionally, matrilinear hare haplotypes were analyzed in order to detect potential familial susceptibility to EBHSV. Sixty-three of 86 sera contained antibodies against EBHSV, whereas 15 of 85 liver samples were PCR positive. Of the latter, 14 were sequenced, revealing three new strains of EBHSV. Fifteen different matrilinear haplotypes were identified, but no correlation was found between haplotype and susceptibility to EBHSV infection. Our findings confirmed the existence of EBHSV in Slovakia and reinforce the need for determining EBHSV status when importing hares for restocking.

Key words: European brown hare syndrome virus, *Lepus europaeus*, phylogenetic analyses, seropositive reactors, Slovakia.

INTRODUCTION

European brown hare syndrome (EBHS) is caused by caliciviral infection of free-living and farmed hares, mainly characterized by acute hepatitis and hemorrhage of the internal organs (Poli et al., 1991). European brown hare syndrome was first diagnosed in Sweden in the early 1980s (Gavier-Widen and Mörner, 1989). In the late 1980s, Lavazza and Vecchi (1989) found viral particles in European brown hares that had died from EBHS and characterized them electron-microscopically. These particles are small icosahedral, nonenveloped, and hemagglutinating single-stranded-RNA viruses (Gavier-Widen and Mörner, 1991), now classified as a calicivirus of the genus *Lagovirus* (Green et al., 2000). This classification was confirmed by cloning and sequencing (Wirblich et al., 1994). European brown hare syndrome virus is highly contagious and transmission usually occurs directly or

indirectly by oral–fecal routes (Morisse et al., 1991). The virus has been found in many European countries (Eskens and Volmer, 1989; Gavier-Widen and Mörner, 1989; Henriksen et al., 1989; Lavazza and Vecchi, 1989; Okerman et al., 1989; Chasey and Duff, 1990; Morisse et al., 1990; Salmela et al., 1993; Steineck and Nowotny, 1993; Gortazar and de Luco, 1995; Frölich et al., 2001) and in Argentina (Frölich et al., 2003). Despite the fact that Slovakia is one of the major exporting countries of European brown hares, information on the occurrence of EBHSV was not available (Slamečka et al., 1997).

The objectives of this study were 1) to determine whether European brown hares from Slovakia were infected with EBHSV and 2) in the case of positive results to analyze any detected EBHSV strain. In addition, we compared results among different locations within south-west Slovakia and among different brown hare haplotypes. This information is of

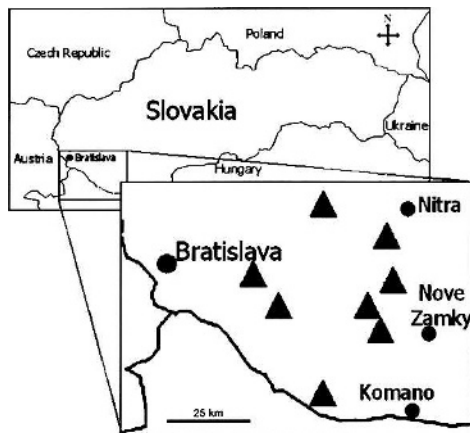


FIGURE 1. Investigation sites (▲) in southwest-ern Slovakia.

practical importance because Slovakia is one of the most important hare-exporting countries. Although most restocking areas in Europe are endemic for EBHSV, the status of source populations should be considered by EBHSV-free countries.

MATERIALS AND METHODS

Samples were obtained from 135 yearlings and adult European brown hares (53 males, 68 females, 14 unknown) shot at eight hunting sites in southwest Slovakia (Fig. 1) in December of 2001 and 2002. Samples were randomly selected from the hunting bag. All sites are located in the Danubian Lowland, which has the highest abundance of European brown hare in Slovakia with the following estimated hare densities: Trnava (48°22'12.3"N, 17°35'17.73"E), 64 hares/100 ha ($n=22$); Neded (48°3'45.04"N, 17°58'17.1"E), 68 hares/100 ha ($n=21$); Tešedikovo (48°13'56.4"N, 17°51'38.05"E), 59 hares/100 ha ($n=21$); Hájske (48°17'59.76"N, 17°52'28.94"E), 36 hares/100 ha ($n=17$); Dlhá nad Váhom (48°18'25.07"N, 17°52'11.72"E), 38 hares/100 ha ($n=4$); Zlaté Klasy (48°6'39.62"N, 17°25'38.32"E), no density data available ($n=11$); Lehnice (48°2'51.99"N, 17°28'33.12"E), 43 hares/100 ha ($n=25$); and Čiližská Radvaň (47°50'16.01"N, 17°41'48.49"E) 26 hares/100 ha ($n=14$). Hare densities are estimated annually at the end of March. Hares are counted either by 30–40 hunters covering the whole hunting ground area or by "strip census," in which hares are counted in a selected area and their density is calculated based upon these results. All data are summarized and annually published in the hunting

statistics of Slovakia (Kaštier et al, 2005). To improve readability, we henceforth will refer to these sampling sites as populations.

Samples were immediately stored at -20°C at the Research Institute of Animal Production, Nitra (Slovakia) and later submitted still frozen for virologic evaluation to the Leibniz-Institute for Zoo and Wildlife Research, Berlin (Germany). Corresponding liver and serum samples were available from 36 animals, whereas from 99 animals, because of logistical difficulties during the hunting procedures, only serum ($n=50$) or liver samples ($n=49$) were available. Thus, virologic analyses were performed on 86 serum and 85 liver samples. Sera were stored at -20°C and were tested for specific antibodies against EBHSV. Liver samples were stored at -80°C and were screened for EBHSV RNA. Antibody prevalence and polymerase chain reaction (PCR) results were calculated for the combined sampling periods (2001–02).

Sera were tested for EBHSV antibodies using an antibody-blocking enzyme-linked immunosorbent assay (ELISA) test kit according to Frölich et al. (1996). Sera were tested at three dilutions: 1:10, 1:100, and 1:1000, and analyzed as quadruplicates. Briefly, 50 μl of the diluted serum sample were added to rabbit IgG anti-EBHSV-coated wells followed by 50 μl predetermined dilution of a known EBHSV-antigen dilution; this gave an optical density (OD) value of approximately 1.0 with negative serum. After overnight incubation at 5°C and a washing step, guinea pig anti-EBHSV hyperimmune serum (detecting serum) was added to two of the wells, and the same concentration of normal guinea pig serum to the other two wells per test sample. Following another incubation period of 30 min at 37°C and another washing procedure, the wells were further incubated with rabbit anti-guinea pig peroxidase conjugate (Denmark code P141, DAKO Diagnostik GmbH, Hamburg, Germany). After washing, the fixed enzymes reacted for 10 min with *o*-phenylene-diamine substrate including perhydrol (Sigma-Chemie GmbH, Deisenhofen, Germany). The reaction was stopped with H_2SO_4 and the results were read at 492 nm on a Sunrise RC photometer (TECAN, Grödig/Salzburg, Austria). The mean OD of negative control sera was calculated. Sera that deviated <3 standard deviations (SD) from the mean of negative control sera in a dilution of $\geq 1:10$ were considered antibody positive. Sensitivity and specificity of the antibody-blocking ELISA was 100% in a serum dilution of $\geq 1:4$.

Reverse transcription (RT) followed by PCR was performed to detect EBHSV RNA. Total

RNA from tissue samples was isolated using the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed in 20- μ l aliquots, containing 10–100 ng RNA, 2.5 μ M random hexamer primers, 5 $\times$ M-MLV buffer, 20 U M-MLV reverse transcriptase (Promega, Madison, USA), 2 U RNase inhibitor (Applied Biosystems, Foster City, California, USA), and 1 mM of each dNTP (Boehringer, Mannheim, Germany). The mixture was initially incubated for 15 min at 37 C and, after that, for 30 min at 42 C. After heat inactivation of the reverse transcriptase (5 min, 99 C) the remaining cDNAs were stored at 4 C. Following RT, a PCR (50 μ l) was performed with 10 μ l of the cDNA reaction under the following conditions: 1 U *Taq* polymerase (Qiagen), 10 $\times$ PCR buffer, 0.5 mM MgCl₂ (both Applied Biosystems), 0.5 mM of each dNTP (Boehringer Mannheim), and 10 pmol of each primer. After an initial denaturation period (9 min at 94 C), DNA was amplified in 35 cycles (94 C, 45 sec; 60 C, 45 sec; 72 C, 60 sec). A terminal elongation phase (7 min, 72 C) ended the PCR. Primers were selected from the VP60 gene of EBHSV: HEF 5'-CCGTCCAG-CATTCGTCCTGTCAC-3' (nt 1795–1817) and HEB 5'-CATCACCAGTCCTCCGCAC-CAC-3' (nt 2038–2059) according to Bascunana et al. (1997). Liver specimens from European brown hares (Germany), previously tested positive by EBHSV-antigen ELISA and EBHSV-RT-PCR, were used as positive controls, whereas RNase-free water served as negative control.

Polymerase chain reaction products were purified with the QIAquick PCR purification kit (Qiagen) and directly sequenced with the specific primers (described above) using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems; ABI) according to manufacturer's instructions, followed by fragment separation on a 3100 Genetic Analyzer (ABI). Sequences were aligned with ClustalX (Thompson et al., 1997). The following sequences from public databases were included in phylogenetic reconstructions: U65356–U65369, U65371, U65372 (Nowotny et al., 1997); Z69620 (Le Gall et al., 1996); U09199 (Wirblich et al., 1994); U80981 (Ros et al., 1996, direct submission); and X98002 (Rossi 1996, direct submission). Neighbor-joining trees were generated with Kimura two-parameter distances using MEGA2 (Kumar et al., 2001). The phylogenetic network was constructed using the median-joining (MJ) algorithm in Network (version 4.1; Bandelt et al., 1999). The MJ

network includes all most-parsimonious trees supported by the data and is particularly appropriate for the low resolution encountered in intra- (or closely related inter-) specific data sets.

In order to investigate potential differentiation in matrilinear susceptibility to EBHSV, the 85 liver samples were also used to determine the haplotype (maternal inheritance) of the respective hare. A 413-bp 5'-end fragment of the variable mitochondrial DNA control region (d-loop) was amplified as described previously (Fickel et al., 1999) and sequenced as described above. ClustalX was used to assign haplotypes whose genealogy (Templeton, 1998) was estimated using TCS 1.13 (Clement et al., 2000). Matrilinear population differentiation (F_{ST}) was estimated by analyzing molecular variance as implemented in Arlequin (Schneider et al., 2000); a Bonferroni correction (Rice, 1989) was applied to *P* values in multiple comparisons.

Prevalence and 95% confidence intervals (CI) were calculated for both the presence of antibodies and the PCR results. The antibody prevalences at different locations (or for different haplotypes) were compared using the exact chi-square test. Adjusted standardized residuals were used to identify the locations responsible for significant differences (Everitt, 1977). Samples with a standardized residual >1.96 (or <-1.96) were considered to be above (or below, respectively) the expected range of prevalence. The significance level was generally set to $\alpha=0.05$. The software package SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA) was applied for the statistical calculations.

RESULTS

Antibodies against EBHSV were present in 63 (73%, 95%CI 63–82%) of 86 blood samples. Of the 63 positive sera, 12 (19%) were positive up to a dilution of 1:10, 40 (63%) up to 1:100; and 11 sera (17%) remained positive at dilutions >1:1000. Antibody prevalences ranged from 36% to 90% and significant differences were detected between geographical locations ($P=0.016$); this resulted from the lower than expected prevalence (36%, adjusted standardized residual = -3.0) found in Trnava.

Fifteen of 85 liver samples (18%, 95% CI 10–27%) were positive in RT-PCR.

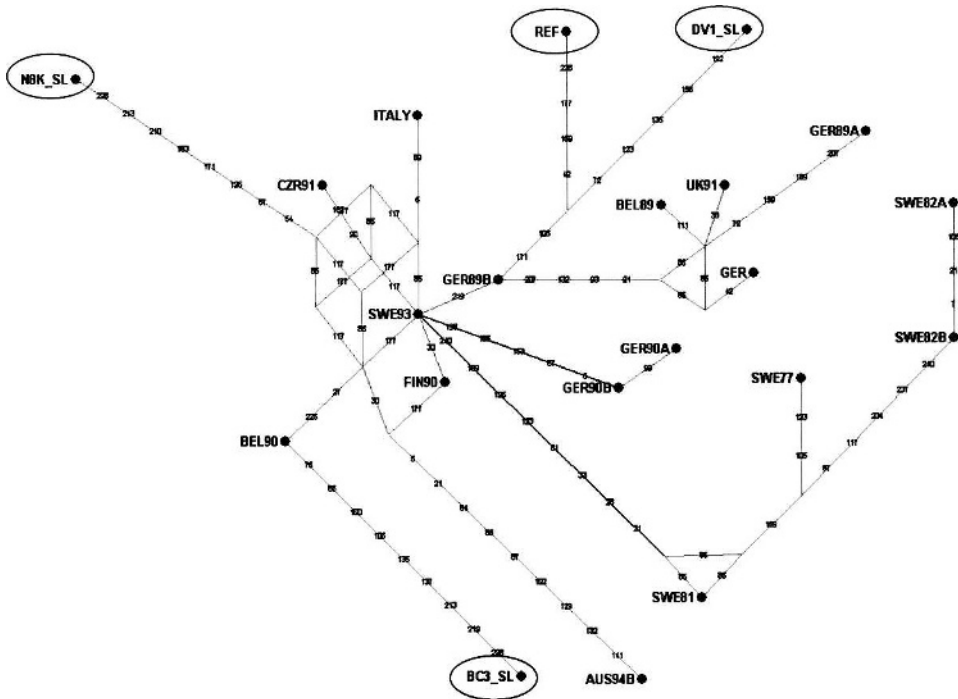


FIGURE 2. Phylogeographic network of EBHSV VP60 sequences. Strains are represented by filled circles with country abbreviation and strain number. Grey numbers along lines indicate mutation sites. Countries of origin are as follows: Ger, Germany (GenBank accession numbers U09199, U65364–67); Swe, Sweden (U65368–71, U80981); Bel, Belgium (U65360–61); Aus, Austria (U65353, U65356, U65358–59); Fin, Finland (U65363); CZ, Czech Republic (U65362); Italy (X98002); UK, United Kingdom (U65372); Ref, reference (France, Z69620). New strains from Slovakia and the reference strain are circled. The length of very long branches has been compressed for drawing purposes.

The positive hares came from the following locations: Lehnice ($n = 8$); Tešedikovo ($n = 4$); Neded, Trnava, and Dlhá nad Váhom ($n = 1$, each). Polymerase chain reaction positive results ranging from 0% to 39% were also significantly different between locations ($P=0.049$), which was because of the higher than expected prevalence in Lehnice (39%, adjusted standardized residual=3.2).

Five of the 15 PCR-positive animals had corresponding serum samples, of which two lacked anti-EBHSV antibodies and three were seropositive with titers ranging from 100 to 1,000. In addition, 14 of all 15 PCR products were sequenced and revealed three new EBHSV-VP60 sequences (GenBank accession numbers: DQ862478–480). One of those three was predominant and carried by 12 individuals (N8k) whereas the two other sequences

(bc3 and DV1) were only found once. The fact that all sequences were easily distinguishable from the reference strain and from the German strain used as positive control (GER, Fig. 2) renders contamination extremely unlikely. Within the 250-bp fragments of the multiple sequence alignments, EBHSV strains Austria92, Austria93, Austria94a, and Belgium90 were identical; thus for subsequent analyses only strain Belgium90 was retained. The alignments of 21 EBHSV sequences (18 from GenBank and the three newly identified Slovakian strains, N8k, bc3, DV1) contained 55 variable sites with 34 phylogenetically informative positions and a transition/transversion ratio of 3.91. Nucleotide distances were calculated for pairwise sequence comparisons of the whole data set. The three new Slovakian sequences differed in their nucleotide

positions from each other in the following manner: N8k–bc3 by 14 positions (pairwise distance $d=0.063$, $SD=0.017$), DV1–bc3 by 17 positions ($d=0.076$, $SD=0.018$), and N8k–DV1 likewise by 17 positions ($d=0.073$, $SD=0.018$). The mean pairwise distance among all 21 sequences was $d=0.050$ ($SD=0.007$), ranging from 0.004 ($SD=0.004$) to 0.109 ($SD=0.023$). The pairwise distances between each new sequence and all others ranged, for bc3, from $d=0.037$ ($SD=0.012$) with Belgium90 (GenBank accession number U65361) to 0.109 ($SD=0.022$) with Sweden82a (U65369); for N8k, from $d=0.046$ ($SD=0.013$) with strain Sweden93 (U65371) to 0.086 ($SD=0.02$) with Sweden82a (U65369); and for DV1, from $d=0.029$ ($SD=0.01$) with strain Germany89b (U65365) to 0.091 ($SD=0.02$) with strain Sweden82a (U65369). A neighbor-joining tree was constructed (figure not shown), but had poor bootstrap support for the clustering of the new sequences and most other branches, except for most Swedish strains. To improve the resolution of related sequences, a phylogenetic network based on variable sites was calculated (Fig. 2). It produced strong evidence that the origin of EBHSV was in Scandinavia (Sweden). Furthermore, network calculations showed that the virus spread from Sweden to western, eastern, and southern parts of Europe.

Mitochondrial DNA (mtDNA) typing of liver samples revealed 15 different haplotypes. Matrilinear population differentiation was very low ($F_{ST}=0.043$) and not significant after Bonferroni correction ($P>0.002$), indicating lack of genetic distance among hares from different sampling sites (panmixia).

In terms of a correlation between matrilinear inheritance (haplotype) and susceptibility to EBHSV infection, no such correlation was found; there was no significant difference detected ($P=0.951$) between the susceptibility of groups of animals with identical haplotypes.

The 15 EBHSV PCR-positive hares

shared six different mtDNA haplotypes: HT02, HT08, HT17, HT65, HT97, and HT99, of which HT02 occurred seven times, HT17 four times, and HT08 twice. All three other haplotypes were only encountered once. However, the high numbers of occurrence of the former three haplotypes are most likely due to the relatively high percentage of occurrence of these haplotypes among the 85 screened hare liver samples (32.9%, 10.6%, and 15.3%, respectively).

DISCUSSION

These results provide the first evidence for EBHSV presence in European brown hare populations in Slovakia. At present, EBHSV has been reported to occur in three other Eastern European countries: Croatia (Sostaric et al., 1991), Poland (Frölich et al., 1996), and the Czech Republic (Nowotny et al., 1997). The number of EBHSV strains identified by molecular techniques is steadily increasing (Nowotny et al., 1997; Chrobocinska, 2000) and there are 24 currently known virus strains (Fig. 2). Within the EBHSV phylogenetic network (Fig. 2) the different EBHSV strains do not group by geographic origin. The only strains clustering along the same branch comprise the samples from Sweden (Sweden93, 81, 77, 82b, 82a; Nowotny et al., 1997). In this study, the sequence analysis revealed a unique position of each of the three new isolates within the taxon EBHSV, not only on different branches, but on their far ends (Fig. 2). Such a lack of clustering indicates that no clear relationship exists between individual strains, time of isolation, or the geographic origin of the isolate. This fact is highlighted by poor bootstrap support for such clustering in the neighbor-joining tree (not shown), demonstrating potentially low homology among the different strains except for the ones from Sweden. These results confirm earlier investigations on phylogenetic relationships among different EBHSV strains (Nowotny et al., 1997).

Most of the reported studies on EBHSV were performed in association with a sudden increase in mortality within the examined hare populations together with post-mortem findings characteristic for EBHSV infection. In contrast, hare populations of the investigated hunting areas of southwest Slovakia seem to be stable in size (Slamečka et al., 2001) and no significant increase in mortality was noted before or during the sampling period. Potential explanation for the apparent lack of associated mortality may relate to increased hare resistance against EBHSV infection or reduced virulence of the Slovakian EBHSV strains. In the study of Trout et al. (1997) on seroepidemiology of the closely related rabbit hemorrhagic disease virus (RHDV), the authors suggest the possible existence of nonpathogenic RHDV or RHDV-like strains. In our study, 63 European brown hares tested seropositive for EBHSV-specific antibodies, among which three individuals also carried EBHSV nucleic acid. It is known that hares succumb to disseminated EBHSV within 2 days postinfection (Lavazza et al., 1997). The fact that we detected EBHSV nucleic acid in association with specific antibodies led us to the hypothesis that a less pathogenic EBHSV variant may exist among European brown hares in Slovakia. This hypothesis is supported by the following studies. Bas-cunana et al. (1997) reported detection of EBHSV by PCR in paraffin-embedded specimens collected in 1977, but the first clinical cases of EBHSV were described 3 yr later in Sweden (Gavier-Widen and Mörner, 1989). Lesions consistent with EBHS have also been described from England since 1976 (Duff et al., 1994) and specific antibodies were found in sera archived since 1962 (Duff et al., 1997); however, confirmed cases of EBHS were first diagnosed by pathologic and electron microscopic (EM) examination in the UK in 1990 (Chasey and Duff, 1990). Moreover, Frölich et al. (2003) found antibodies to EBHSV, EBHSV antigen, and virus

particles characteristic of calicivirus by EM in free-ranging European brown hares from Argentina, but EBHS has not been reported to cause abnormal mortality in these hares.

In addition, the EBHSV transmission rate is affected by host population densities. It is thought that high hare populations may affect (reduce) EBHSV mortality because of increased transmission and resulting infection of hares at a young age (<3 mo), which subsequently results in the development of protective antibodies (Lavazza et al., 1997). The number of seropositive reactors against EBHSV within the local hare populations of the study area varied between 36% and 90%. The lower than expected value from the region of Trnava could indicate a past epidemic peak with decreasing antibody titers. Although information exists regarding seroconversion directly after exposure (Lavazza et al., 1996), to our knowledge, there are no reports concerning the course of antibody titers over longer periods.

To determine whether an inherited susceptibility (Tang et al., 2002) exists among the investigated hares, we also examined the matrilinear haplotypes of all tissue samples ($n=85$). Fifteen different haplotypes were found, but there was neither a significant correlation between haplotype and development of antibodies nor between haplotype and harboring of EBHSV infection. Thus, matriline of the investigated Slovakian hare population revealed neither preferences for nor protection against EBHSV infections. Because haplotype-based analysis of population differentiation indicated panmixia (a sufficient number of migrants among sites), the cause for the local differences in seroprevalence remains ambiguous. The explanation that infected hares did not equally contribute to gene flow seems unlikely, but maybe the source of infection was outside the sampling area and the virus had not yet been spread across all sites.

In conclusion, three new strains of EBHSV were found in European brown

hares from Slovakia, and in certain hunting areas antibody prevalences reached 90% within the hare population. The facts that no significant increase in mortality was noted and that we detected EBHSV nucleic acid in association with specific antibodies led us to the hypothesis that a less-pathogenic EBHSV variant may exist among European brown hares from Slovakia. Haplotypic matriline were not correlated with the occurrence of infection in Slovakian hares, which appeared to be at random. When restocking wild hare populations in EBHSV-free areas, care should be taken regarding the origin of the animals and their possible status as carriers of the virus.

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