European brown hare syndrome in northern Italy: results of a virological and serological survey *

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Summary: Between August 1988 and August 1991, 456 carcasses of captive or sylvatic hares from several areas of northern Italy, and 931 sera taken from adult hares in farms, in hunting and natural reserves and on importation were examined using virological (sandwich enzyme-linked immunosorbent assay [ELISA] and immuno-electron microscopy) and serological (competition ELISA) tests. The epidemiological data presented relate to the incidence of European brown hare syndrome (EBHS) in various provinces of northern Italy, the mortality caused by EBHS and the seasonal frequency of this disease. The endemic character of EBHS in Italy is proved by the large number of samples testing positive for EBHS virus (EBHSV) (47.6%) and by the results of the seroepidemiological survey, in which approximately 95% of samples tested positive for specific anti-EBHSV antibodies, showing varying titres according to the different environmental conditions.

KEYWORDS: Diagnostic methods – European brown hare syndrome – Hares – Italy – Viral diseases.

INTRODUCTION

European brown hare syndrome (EBHS) is a viral disease, the clinical, anatomo-pathological and epidemiological characteristics of which have been described only recently (6, 9, 10, 11, 14, 17, 18). At present, EBHS is reported as endemic in several European countries (5, 7, 8, 10, 13, 19, 20, 22) and the disease may have already been present in most of these countries for the past ten years (6, 12, 20, 23).

Even in Italy, where the first secure diagnosis of EBHS coincided with the definition of its viral aetiology (14), the disease seems to have been widespread since the beginning of the 1980s (23, 24).

The renewed interest in EBHS arises partly from the numerous similarities with another disease, recently introduced into Europe in both wild and domestic rabbits, known as rabbit viral haemorrhagic disease (VHD) (16, 20, 21). The research conducted at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia since the

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first description of the aetiological agent of EBHS have followed a number of different directions:

a) chemical/biological characterisation of the viral agent, allowing this to be classified, along with the VHD agent (VHDV), within the Caliciviridae family (1, 3, 21)

b) study of the antigenic properties of the viral agent and correlations with VHDV, also using monoclonal antibodies (MAb), leading to consideration of the two agents as distinct serotypes with common antigenic determinants (1, 2, 3, 21)

c) reproduction trials and cross-infection experiments employing EBHSV and VHDV, excluding the hypothesis of an interspecific transmission of the two viruses (3; Scicluna, unpublished findings)

d) preparation of diagnostic reagents used in both virological diagnostic methods (i.e. sandwich ELISA, immuno-electron microscopy [IEM] and Western blotting) and serological methods (e.g. competition ELISA) (3, 4, 14, 15, 21, 25)

e) examination of diagnostic samples (carcasses and sera) in the laboratory, with the dual aim of verifying the specificity and sensitivity of the diagnostic tests and evaluating the incidence and diffusion of the disease, mainly within Lombardy and Emilia Romagna (the territory covered by the jurisdiction of the Institute).

MATERIALS AND METHODS

Animals

Figure 1 shows the regions from which hare carcasses and sera used in this study were taken.

Between August 1988 and August 1991, 456 hare carcasses, almost all (90.1%) of which came from northern Italy (33.3% from Lombardy and 56.8% from Emilia Romagna) were examined. In most cases (72.6%), these were wild hares killed or found dead in game reserves or in protected areas, while the rest (27.4%) came from 77 farms where they were reared in cages or in runs (Table I).

Virological investigations

Virological examination for EBHSV was conducted, using both sandwich ELISA and negative-staining IEM, on liver and/or spleen homogenates of all the hares given to the diagnostic sections of the Institute, regardless of whether lesions indicative of EBHS were present. The ELISA used an anti-VHDV rabbit hyperimmune serum as capture antibody and a pool of conjugated MAb (produced towards VHDV but cross-reactive with EBHSV) as detection antibody (3, 4). Observation by IEM was performed on liver homogenates which were incubated with anti-VHDV hyperimmune serum, centrifuged at very high speed and then stained negatively with 2% sodium phosphotungstate (3, 24).

Sera

A total of 773 sera was collected from different areas while restocking was occurring in the game reserves. Of these sera, 76.45% were taken from hares captured during restocking campaigns and 23.55% from hares reared in captivity (Table I). A second batch of serum samples was collected from hares imported from Hungary (108 sera) and Argentina (50 sera), before these animals were set free in hunting areas in the provinces of Brescia and Pavia.
Map of part of Italy, showing the areas covered in this study (see Table I for the names of regions; shaded area corresponds to the territory under the jurisdiction of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia in Brescia)

Serological investigations

All sera were assayed for anti-EBHSV antibodies by competition ELISA. After adsorbing an anti-EBHSV hyperimmune serum, the serum sample was incubated on the sensitised plate, together with a partially-purified EBHSV antigen, and conjugated immunoglobulin G purified from the same hare serum was used as detection antibody (3, 15).

Simultaneously, a study was conducted to verify the degree of correlation between the anti-EBHSV and anti-VHDV titres. The latter was obtained in an ELISA similar to
TABLE I

Geographical distribution of virological and serological testing for European brown hare syndrome virus in hares in Italy.

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of hares examined*</th>
<th>Origin of sera</th>
<th>No. of hares*</th>
<th>No. of farms</th>
<th>No. of hares testing positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Lombardy</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Bergamo (BG)</td>
<td>12 (10)</td>
<td>-</td>
<td>-</td>
<td>2</td>
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</tr>
<tr>
<td>Brescia (BS)</td>
<td>32 (19)</td>
<td>32 (0)</td>
<td>3</td>
<td>12</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Como (CO)</td>
<td>ND ND</td>
<td>ND ND</td>
<td>ND ND</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Cremona (CR)</td>
<td>7 (4)</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>3 (3)</td>
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<tr>
<td>Mantova (MN)</td>
<td>19 (13)</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>6 (6)</td>
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<tr>
<td>Milan (MI)</td>
<td>17 (17)</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>6 (6)</td>
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<td>Pavia (PV)</td>
<td>49 (30)</td>
<td>57 (0)</td>
<td>9</td>
<td>26</td>
<td>16 (16)</td>
</tr>
<tr>
<td>Sondrio (SO)</td>
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<td>-</td>
<td>-</td>
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<td>Varese (VA)</td>
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<td>-</td>
<td>-</td>
<td>3</td>
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</tr>
<tr>
<td>Sub-total</td>
<td>152 (101)</td>
<td>89 (0)</td>
<td>12</td>
<td>62</td>
<td>35 (35)</td>
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<td>Emilia Romagna</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bologna (BO)</td>
<td>36 (29)</td>
<td>179 (179)</td>
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<td>19</td>
<td>16 (16)</td>
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<td>Ferrara (FE)</td>
<td>103 (86)</td>
<td>251 (216)</td>
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<td>57</td>
<td>46 (46)</td>
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<td>Forli (FO)</td>
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<tr>
<td>Modena (MO)</td>
<td>11 (11)</td>
<td>9 (0)</td>
<td>1</td>
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<tr>
<td>Parma (PR)</td>
<td>43 (25)</td>
<td>135 (135)</td>
<td>0</td>
<td>22</td>
<td>11 (11)</td>
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<td>Piacenza (PC)</td>
<td>35 (27)</td>
<td>48 (48)</td>
<td>0</td>
<td>17</td>
<td>12 (12)</td>
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<tr>
<td>Ravenna (RA)</td>
<td>5 (2)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0 (0)</td>
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<tr>
<td>Reggio Emilia (RE)</td>
<td>23 (20)</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Sub-total</td>
<td>259 (202)</td>
<td>635 (591)</td>
<td>5</td>
<td>126</td>
<td>91 (91)</td>
</tr>
<tr>
<td>Other provinces</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Alessandria (AL)</td>
<td>- -</td>
<td>9 (0)</td>
<td>1</td>
<td>- -</td>
<td></td>
</tr>
<tr>
<td>Cuneo (CN)</td>
<td>6 (0)</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Naples (NA)</td>
<td>9 (1)</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Padova (PD)</td>
<td>2 (2)</td>
<td>16 (0)</td>
<td>2</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pisa (PI)</td>
<td>24 (24)</td>
<td>12 (0)</td>
<td>1</td>
<td>19</td>
<td>19 (19)</td>
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<tr>
<td>Rome (RM)</td>
<td>1 (1)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Treviso (TV)</td>
<td>3 (0)</td>
<td>12 (0)</td>
<td>1</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sub-total</td>
<td>45 (28)</td>
<td>49 (0)</td>
<td>5</td>
<td>29</td>
<td>20 (20)</td>
</tr>
<tr>
<td>Total</td>
<td>456 (331)</td>
<td>773 (591)</td>
<td>22</td>
<td>217</td>
<td>146 (146)</td>
</tr>
</tbody>
</table>

* figures in brackets represent wild hares
ND: no data
that performed for EBHSV, but using reagents specific for VHDV, namely a hyperimmune anti-VHDV serum as capture antibody, and one semi-purified VHDV and one MAb specific for VHDV as detection antibody (3, 4, 25).

RESULTS AND DISCUSSION

The virological examination of the 456 hares yielded positive results for EBHSV in 217 cases (47.6%). Of the total number of hare sera testing positive, 67.3% were from wild animals, while the remainder were from outbreaks on 39 different farms (Table I).

The necroscopic examination established a correlation between virological detection of EBHS and the following lesions, which could be considered typical of the disease: tracheal and lung congestion; oedema; hepatic congestion and degeneration; splenomegaly; presence of uncoagulated blood in the body cavities; and jaundice.

The overall rate of positivity was obtained by using both IEM and ELISA results. The two methods (for 335 diagnostic samples) agreed in 93.4% of cases.

A comparison of the seasonal evolution of the mortality of hares and the total number of EBHSV-positive samples indicates that the major incidence of the disease was registered in October, November and December (Fig. 2a). Regardless of the

![FIG. 2a](image)

Seasonal evolution of mortality in hares compared to the European brown hare syndrome virus-positive virological findings in Italy between August 1988 and August 1991
environmental conditions and the more careful control of the territory during this period (both of which facilitated carcass recovery), the trend of the disease was almost identical in wild hares and in hares reared in captivity (Fig. 2b). Therefore, although EBHS is present almost throughout the year, the disease is more frequent in the autumn months. This could be due to the presence of a higher number of susceptible animals, i.e. breeders plus all the animals born during the year. In view of a number of factors—EBHS is principally transmitted directly (although indirect transmission also occurs), no insect vectors seem to be involved, and hares aged 2-3 months contract the infection without developing the disease—it may be argued that ‘density’ is an important conditioning element in the epidemiology of this disease. Activities such as hunting and restocking of hares may also aid viral dissemination.

Results of the serological investigation conducted on sera from wild hares were expressed as a percentage and classified according to titre (Fig. 3a). Distribution of these results was similar for each province, yielding overall data which were practically identical throughout the region (Fig. 3b). The most representative groups were those with low to average values ($\leq 1/160$ and $1/320-1/640$); these two groups accounted for 41.79% and 34.69% of results, respectively. In addition, a high titre of $1/1,280-1/2,560$ or $\geq 1/5,120$ was recorded for a considerable number of sera (17.43% and 5.24%, respectively). Similar titres were also observed in captive hares which were recovering from the disease. Very few sera (0.85%) from sylvatic hares yielded entirely negative results: these were the only animals considered susceptible to EBHS.

![Comparison of seasonal evolution between European brown hare syndrome outbreaks in captive and in wild hares in Italy between August 1988 and August 1991](image-url)
The overall serological data indicate the endemic nature of EBHS in all provinces investigated, thus providing ulterior confirmation of the virological results described above.

The 182 sera from captive hares (from 22 different farms in 8 provinces) were again classified according to titre: 27.48% gave negative results; 45.60% presented low titres (≤1/160); 19.78% had average titres (1/320-1/640) and only 7.14% had high titres (1/1,280-1/2,560 and ≥1/5,120). A number of differences can be observed in relation to the titres obtained for wild hares, the most outstanding being the higher percentage of seronegative captive hares (Fig. 3b). The fact that the sanitary condition of captive hares was usually known made it possible to analyse the serological results from these animals in relation to this sanitary data, as revealed by the history of the farm and possible virological findings. In fact, the farms concerned were divided into four categories, each corresponding to a different sanitary situation:

a) In 6 farms (a total of 39 hares), no anti-EBHSV antibodies were observed, indicating complete susceptibility of the animals to the disease. This situation usually occurs when the management of the farm guarantees limited or no contacts between the animals and the outside world.

b) In 5 farms (48 hares), where cases of EBHS were diagnosed by virological findings prior to serological testing, serum titres were average to high (between 1/320 and 1/50,000). Titres varied according to the time which had elapsed since the last case of
EBHS, the age of the survivors during the outbreak, and whether animals had been newly introduced.

In the remaining 11 units (95 hares), an average or low titre (<1/320) was registered.

c) In certain cases, the history of the farm referred to past outbreaks of disease resembling EBHS. This was considered as justifying the presence of seropositive animals.

d) Conversely, in other units – even in the presence of (mostly low) positive titres (1/20-1/80) – the sanitary history of the farm excluded past episodes of the disease.

Similarly, Smid et al. (26) reported the presence of low titres of anti-VHDV antibodies in rabbit sera in former Czechoslovakia, some of which had been collected prior to the first diagnosed cases of VHD in Europe. Smid et al. (26) suggested that the antibodies could have been 'naturally acquired' due to the existence in lagomorphs of one or more viral strains which are antigenically correlated to VHDV, and in this case to EBHSV, but which do not present pathogenic activity.

More than 100,000 hares are imported into Italy each year, and these animals are mixed with the local hares used in the restocking of hunting areas, causing a continuous redistribution throughout the territory of animals with different levels of potential exposure to EBHS. This justified the serological investigation conducted on hares imported from Hungary and Argentina.

It is difficult to draw any precise conclusions from the results obtained, due to the limited number of sera examined and the fact that neither Hungary nor Argentina has
ever reported a case of EBHS. In fact, the distribution of titres in these sera is comparable to the situation observed in northern Italy (Fig. 3b). The serological positivity observed in hares from Hungary may have been due to the free movement of wild animals, as Hungary shares borders with two infected countries (Austria and former Czechoslovakia). The results obtained for hares from Argentina are surprising, as there are no reports of EBHS anywhere in the Americas. These results could again be explained by the possible existence of apathogenic correlated viral strains.

Finally, an additional aim of this research was to study the relationship between anti-EBHSV and anti-VHDV titres given by the same hare sera. The titre for EBHSV was constantly higher than for VHDV in all sera, and the ratio between the two sets of titres depended on the value of the EBHSV titre. When the titres were low (1/40-1/160), the ratio was between 4 and 16, while for titres considered as average (1/320), this ratio varied between 8 and 64, reaching values of between 16 and 512 for the highest titres (>1/320). These results represented further confirmation of the significant antigenic differences between EBHSV and VHDV (2, 3, 15).

**CONCLUSIONS**

The results of this investigation of EBHS in northern Italy over the last three years proved the specificity and sensibility of the diagnostic tests employed. At present, the information available on the aetiological agents of EBHS and VHD, and in particular on the biochemical and antigenic characteristics of these two diseases, ensures a differential diagnosis, both in organ homogenates and in sera from hares and rabbits.

EBHS should no longer be regarded as a disease of recent introduction, but rather as the most important disease of hares; the presence of EBHS is independent of the diffusion of VHD among domestic and wild rabbits. EBHS is highly contagious, inducing high rates of morbidity and mortality, and occurrence is widespread among both wild and captive animals throughout northern Italy. The economic losses due to EBHS are difficult to estimate, but the environmental damage is evidently vast in terms of the ecology of the wild fauna of the affected regions.

The epidemiological characteristics of EBHS have not been clearly defined to date, and further studies should be conducted to determine which countries are to be considered infected, and to ascertain the nature of the diffusion of the disease, the pathogenetic mechanisms of the infection, and the natural transmission routes.

At present, control of EBHS is only possible through the application of programmes of serological and virological survey accompanied by sanitary measures (the latter being necessarily limited when wild animals are involved). Indirect control of the disease has not yet been achieved by the use of a vaccine, due to the impossibility of isolating EBHSV on cell cultures and the ineffectiveness of the VHD vaccine in protecting hares from EBHSV infection (S. Barei, personal communication).

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Resumen: Entre agosto de 1988 y agosto de 1991, 456 cadáveres de liebres en cautiverio o silvestres de varias regiones del norte de Italia y 931 muestras de sueros extraídas de liebres adultas procedentes de criaderos, reservas naturales o territorios de caza, así como también de animales importados, se sometieron a pruebas virológicas (prueba inmunoenzimática [enzyme-linked immunosorbent assay: ELISA] «sandwich» y microscopía electrónica) y serológicas (prueba ELISA de competición). Los autores presentan los datos epidemiológicos así obtenidos sobre la incidencia del síndrome de la liebre pard europea (European brown hare syndrome: EBHS) en distintas regiones del norte de Italia, la mortalidad causada por esta enfermedad y su frecuencia según las estaciones. Concluyen que el síndrome es endémico en Italia, según lo revelan la gran cantidad de muestras que incluían el virus del EBHS (47,6 %) y los resultados de la investigación seroepidemiológica, ya que 95 % de los sueros tenían los anticuerpos específicos del virus, con titulación diversa según las condiciones del medio ambiente.


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REFERENCES


