Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome *

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Summary: Development of methods for the diagnosis of viral haemorrhagic disease and the European brown hare syndrome has proceeded at a steady pace over the last few years. The studies conducted by the authors demonstrate that, like VHDV, EBHSV is a calicivirus. The degree of correlation between the two viruses is a key question both for understanding their biology and interpreting the diagnostic results. A discussion of the similarities and differences between VHD and EBHS is followed by the presentation of the latest antigenic correlation results of the two viruses. Considering the absence of culture procedures to isolate either virus, the diagnostic methods discussed in this review are the haemagglutination (HA) test, immune electron microscopy (IEM) and enzyme-linked immunosorbent assay (ELISA) for virus detection, and the haemagglutination inhibition test (HI) and ELISA for antibody detection. The major obstacles, especially for the diagnosis of EBHS, are described; these are represented by morphological, structural and antigenic modifications due to proteolytic degradation. A differential diagnostic method for the two diseases, based on MAb ELISA, is presented. A final conclusion, drawn from the epidemiological analysis of the virological and serological data, is that EBHS and VHD should be considered as two distinct diseases, each caused by its own aetiological agent.


INTRODUCTION

The diagnosis of an outbreak of viral haemorrhagic disease (VHD) of rabbits has, until recently, been considered less problematic than many other aspects of this disease. In fact, the peculiarity of the epidemiological features, clinical signs and pathological changes of VHD often make a provisional diagnosis of VHD fairly simple. Furthermore, because VHD virus (VHDV) can agglutinate type “O” human erythrocytes, the haemagglutination (HA) test as well as the haemagglutination inhibition (HI) test have proved to be easy laboratory techniques, useful, respectively,
for the detection of VHD antigen and of its specific antibodies (56). Since the first outbreaks reported in the People's Republic of China (37), both methods have been widely applied in many laboratories as routine diagnostic tests of VHD.

At present, the epidemiological situation of all the countries in which VHD has been notified indicates its endemic character (1, 19, 38, 45, 60). Although spread of the disease helps in diagnosis, future applications of the disease control programmes, based on health regulations and vaccination and/or eradication schemes, may result in modifications of its epidemiological behaviour. These changes would probably hinder VHD clinical diagnosis, which would then require more reliable laboratory methods.

The clinical diagnosis of the European brown hare syndrome (EBHS) is more problematic, particularly when the disease occurs in wild hares rather than in hare breeding units, where the pathological evolution is similar to VHD. The carcasses of wild hares are only occasionally discovered; because they are usually in an advanced state of decomposition, a correct interpretation of possible lesions is difficult. Another difficulty relates to the identification of the aetiological agent; as described later, this problem necessitates the use of more refined diagnostic techniques, such as immune electron microscopy (IEM).

When preparing a review on the diagnosis of a new disease, the major difficulty encountered is the limited number of references available in literature. Although the articles published on VHD give a fairly detailed description of its various features, the information available on EBHS is still fragmentary and, in part, contradictory (10, 32, 34). Of the latter disease, little has yet been reported apart from its clinical evolution, its gross and histopathological lesions and its aetiological agent, only recently acknowledged as a virus (17, 24, 33).

From the many similarities between VHD and EBHS with respect to the epidemiological evolution, symptomatology and pathological lesions of the two diseases, the question of the degree of correlation and possible identity of the viruses logically arises (33). The answer to this question would obviously be helpful in determining the type of control and eradication campaigns to be adopted with respect to domestic and wild animals.

This situation has led the authors to present, as an introductory description of the diagnostic procedures of the two pathologies, the results of their experimental studies. The primary interest of the authors has been to characterise the structural, biochemical and antigenic properties of the aetiological agent of EBHS (EBHSV), and particularly to establish the degree of correlation with VHDV to promote the correct use of new diagnostic techniques.

CORRELATIONS BETWEEN EBHS AND VHD

The evidence of the clinical and pathological similarities which exist between the recently reported cases of VHD and EBHS (24) encouraged many research workers to conduct more thorough studies of EBHS, which has been present in Europe at least since the early 1980s. Investigations of EBHS were undertaken by the authors. These investigations shed new light on the viral aetiology of EBHS (Gavier, personal
communication; 9, 33) and have led to the diagnosis of outbreaks in various European countries, including Denmark (14, 28), Belgium (51), Yugoslavia (Paukovic, personal communication) and Great Britain (12).

The principal pathological features common to both diseases were found to be: rapid evolution, mild nervous symptoms, degeneration and congestion of the liver, the presence of serohaemorrhagic liquid at the nostrils, congestion of the spleen and kidneys, the presence of uncoagulated blood in the body cavities, diffuse or petechial haemorrhages on serosa and mucosa and the occasional presence of jaundice. Other similarities included extremely high morbidity and mortality rates and preferential susceptibility of adult animals, especially breeders, while animals under the age of 40 days remained unaffected (17, 18, 25, 29, 33, 41).

The complete morphological identity and the antigenic correlations of the viral particles, as demonstrated by IEM, have led some workers to believe that VHD and EBHS are the same disease (41).

Nevertheless, a comparison between the epidemiological situation and the geographic distribution of outbreaks in the two species suggests a need for more caution. In fact, the high incidence of disease in one species in situations where both species were present was significant in itself. An example of such a situation was reported in Great Britain, where numerous cases of EBHS have been notified without ever diagnosing VHD (12); and in Sweden (25) and Denmark (29, 59), the disease in rabbits was reported several years after the first outbreaks of EBHS. On certain Italian farms where both species were bred, only hares were diagnosed as diseased (Capucci and Scicluna, personal observations). Attempts to solve the problem by conducting experimental cross-reproductions have given contrasting results. In France, Morisse et al. (46) transmitted the disease to rabbits, inoculating them with EBHSV-positive hare organ suspensions. In Italy, Di Modugno and Nasti (15) transmitted the disease to two of nine hares which had been inoculated with VHDV-positive rabbit organ suspensions and again reproduced the disease in rabbits, after inoculating them with organ homogenates from the two deceased hares. By contrast, when the authors recently conducted an experiment in their laboratory and inoculated hares and rabbits with EBHSV-positive hare extracts, the disease was only reproduced in the hares, while the inoculated rabbits remained healthy and did not seroconvert; when subsequently challenged with VHDV, however, they died within the first 48 h post infection (p.i.) (Capucci and Scicluna, personal observations). Furthermore, Ronsholt et al. (59) reported the impossibility of transmitting the disease in hares inoculated with VHDV-positive rabbit organ suspensions.

At present, there are no explanations for the contrasting results of certain of these reported cross-reproduction trials. The problem will certainly be solved when more knowledge of the pathogenesis of each disease is acquired and more precise and analytical virological and serological methods are applied as they have been, in part, in the biological trials conducted by the authors. Among the laboratory data indicating the close relationship of the two viruses, which are both members of the Caliciviridae family, are the inability of either virus to grow in vitro, their similar morphological features and the biochemical discovery of a single principal structural protein for EBHSV, with a weight identical to that of VHDV (9). At this point, it is necessary to provide a detailed study of the antigenic characteristics of the two viruses in order to establish the degree of correlation between them.
ANTIGENIC STUDIES OF EBHSV AND VHDV

Antigenic correlation studies of the viruses were carried out by the authors in their laboratory using convalescent and hyperimmune rabbit anti-VHDV sera, convalescent hare anti-EBHSV sera and monoclonal antibodies (MAb) produced against VHDV. Crude organ extracts, similar in preparation to the diagnostic samples, and virus from different stages of purification were the antigenic sources of the two viruses (8, 11). The investigations conducted on VHDV were easier and more accurate than those carried out on EBHSV. One reason for this was the more constant availability of material for the study of VHDV, because of its relatively simple experimental reproduction, while the main supply of EBHSV were field samples submitted for diagnosis. As a consequence, the use of a reference virus strain was possible during research studies only in the case of VHDV. In addition, the viral yield after each purification step was always notably higher for VHDV than for EBHSV so that the production and control of specific immunological reagents necessary for the study of VHDV were more easily achieved.

The authors principally used two immunological techniques in their investigations. One was the ELISA sandwich, similar to the one for the virological diagnosis which will be described later. Several immunological reagents were used for adsorption and detection according to the different purposes of the studies described. The other was the Western blot technique (WB), made possible by the ability of two MAb (5D1 and 5G3) to detect, with a high degree of efficiency and specificity, the structural protein (VP60) of both viruses (8, 11).

HA, IEM and ELISA were used for the viral diagnosis of VHD. Analysis of the results obtained showed that about 5% of the positive samples were HA-negative (VHDV/HA-negative) but highly reactive in the other two methods and clearly antigenically distinguishable from the “normal” VHDV (VHDV/HA-positive). A parallel WB analysis of VHDV/HA-positive and VHDV/HA-negative samples helped clarify this behaviour (Fig. 1, lanes “a” and “d”, respectively). Lane “d” in Figure 1 illustrates the proteolytic degradation of the VP60 observed in the VHDV/HA-negative sample: the viral protein appears to be cleaved to polypeptides having a molecular weight of less than 40 kDa; the two main polypeptides, detected by MAb 5D1, weigh approximately 26-28 kDa. The same modification was observed in EBHSV (Fig. 1, lanes “b” and “c”), with two differences: first, the higher percentage of samples which underwent degradation, up to about 50%, and second, the presence of VP60-related polypeptides, reactive with MAb 5D1, weighing slightly less than those revealed for VHDV.

The origin and nature of the proteolytic activity observed in both viruses are still obscure; this might be due to the autolytic processes which take place in the carcass or to the physiological alterations of the host, which may be connected to the pathogenetic evolution of the disease. In any case, if the differences in morphology visualised by EM (Figs. 2 and 3) and the profound antigenic changes (described further on) are considered, the dramatic modifications in the viral surface caused by the proteolytic cleavage of VP60 are evident. Such alterations not only affect the diagnostic results of the two diseases, but also the correlation studies of the two viruses. Similar phenomena have also been observed in other members of the Caliciviridae family (30). An interesting example is the aetiological agent of human hepatitis type E (2), whose principal structural protein undergoes similar proteolytic degradation; as stated
by Bradley and Balayan (3), the confounding effects of degradation should be taken into consideration when studying the morphology and serology of the virus particles.

FIG. 1

Western blot analysis of VHDV and EBHSV using MAb 5D1

Lanes "a" and "d" illustrate the analysis conducted on two consecutive 3-fold dilutions of two VHDV-positive rabbit liver diagnostic samples. The HA-positive sample is presented in "a", the HA-negative sample in "d". Note that in "d" the VP60 is totally degraded in polypeptides inferior to 40 kDa, weighing principally 26-28 kDa. In lanes "b" and "c" are presented three consecutive 4-fold dilutions of two different EBHSV-positive hare liver samples, previously concentrated (× 20) by ultracentrifugation. Again, it is possible to demonstrate the proteolytic cleavage of the VP60, less than 10% in "b" and more than 90% in "c", in fragments weighing principally 23-26 kDa. The molecular weights described are indicative.

Nearly 50 MAb were obtained from three successive fusions. Table I summarises the principal characteristics of the eight MAb purposely selected for the diagnosis of the two diseases and for the study of the antigenic correlations of their aetiological agents. It is important to add that Group I, corresponding to the VHDV-specific MAb, was composed of approximately 80% of the total MAb produced, while Group II consisted of the remaining 20% cross-reactive MAb. Besides their specificity, Table I also shows data on a functional aspect of the MAb; i.e. their capacity to neutralise the virus in vivo. Among the MAb tested, only those which were VHDV-specific possessed this fundamental property, thus indicating that the area directly implicated in the infection/protection mechanisms is antigenically different for the two viruses.
FIG. 2

Electron micrograph of VHD viral particles from a purified rabbit liver extract

By negative staining (2% sodium phosphotungstate, pH 7.2)
Scale bar = 100 nm

A) Most particles show 5-fold axis: a central patch surrounded by a rim from which radiates a fringe of ten short and evenly spaced "spikes"

B) One particle (full arrow), viewed along the 3-fold axis of symmetry, shows the characteristic cup-like depressions set in a "star of David" pattern. Another particle (empty arrow) is viewed along the 2-fold axis

C) Aspect of virions after proteolytical degradation: no structures are visible on surface of particles, which seem smaller (23-25 nm) and are outlined by the rim of the core
**FIG. 3**

Electron micrograph of EBHS viral particles from a purified hare liver extract

By negative staining (2% sodium phosphotungstate, pH 7.2)
Scale bar = 100nm

A) Small group of full and empty virions, morphologically similar to VHDV illustrated in Fig. 2A

B) 23-25 nm smoothed particles after proteolytical degradation
TABLE I
Schematic representation of the principal characteristics of the anti-VHDV MAb employed in diagnostics

<table>
<thead>
<tr>
<th>Group</th>
<th>MAb</th>
<th>VHDV</th>
<th>EBHSV</th>
<th>VP60</th>
<th>Surface exposition</th>
<th>VHDV in vivo protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1H8</td>
<td>P</td>
<td>A</td>
<td>-</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>I</td>
<td>3H2</td>
<td>P</td>
<td>A</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>6F9</td>
<td>P</td>
<td>P</td>
<td>-</td>
<td>+/-</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>3H6</td>
<td>P</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>6G2</td>
<td>P</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>6D6</td>
<td>P</td>
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<td>II</td>
<td>5G3</td>
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<td>P</td>
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<td>ND</td>
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<tr>
<td>II</td>
<td>5D1</td>
<td>P</td>
<td>P</td>
<td>+</td>
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<td>-</td>
</tr>
</tbody>
</table>

P = Presence of epitope  
A = Absence of epitope  
ND = Not defined

The VP60 reactivity with all MAb, studied in Western blotting, had identical results for both viruses.

Surface exposition refers to the availability for antibody binding of the epitopes on the intact structure of the virus.

VHDV protection refers to the in vivo viral neutralisation trial conducted in collaboration with Dr Ronsholt (State Veterinary Institute for Virus Research, Lindholm, Denmark).

1H8 is an excellent neutralising MAb since it prevents clinical evolution of the disease and completely abolishes the immunological response towards VHDV.
In the ELISA correlation studies, convalescent and hyperimmune sera of both species were used as catchers and horseradish peroxidase (HRP) conjugated MAb or polyclonal IgG, purified from the previous sera, were used as tracers. The results of the ELISA sandwich for the VHDV and the EBHSV are reported in Figure 4. The polyclonal hare anti-EBHSV serum shows little binding with VHDV, either as catcher or as tracer; those MAb reacting with internal epitopes (cross-reactive MAb) are negative with the purified VHDV (Fig. 4A) but, owing to the presence of viral subunits (Fig. 4B), positive with the crude extract. Figure 4C illustrates the reaction of the same VHD/HA-negative extract which was shown in WB to contain degraded virus (Fig. 1, lane “d”). Following the morphological and biochemical modifications induced by the proteolytic degradation, the profound changes in antigenicity, principally within the VHDV-specific area, become noticeable. The main consequence of the VP60 cleavage is indicated in Figure 4C:

1) a sharp drop in the reactivity of all the MAb with epitopes located on the viral surface, particularly evident for the major neutralising MAb 1H8;

2) the simultaneous increase in reactivity of the anti-EBHSV serum, which became as positive as the anti-VHDV serum.

At least in part, this antigenic picture appears comparable to that of the EBHSV-positive samples (Fig. 4D) because of the already indicated specificity of MAb 1H8 and 3H2 and the overall reactivity of the polyclonal sera. A prevalent positivity for EBHSV is, in fact, shown by the homologous serum, although less clearly than for the VHD system.

**FIG. 4**

**ELISA sandwich reaction of VHDV and EBHSV employing MAb and polyclonal sera**

The solid phase is sensitised either with rabbit anti-VHDV serum (full bar) or with hare anti-EBHSV serum (empty bar). MAb, rabbit anti-VHDV (VHD) IgG and hare anti-EBHSV (EBH) IgG, all directly conjugated to HRP, have been employed as tracers. The samples examined are, respectively: “A” a purified form of VHDV; “B” a VHDV HA-positive rabbit liver extract; “C” a VHDV HA-negative rabbit liver extract; “D” an EBHSV-positive hare liver extract. The abscissa represents the values expressed as absorbance at 492 nm.
From the experimental results available, it is possible to establish a preliminary estimation of the degree of cross-reactivity between the two viruses. Although an antigenic correlation between the two aetiological agents has been demonstrated, this correlation is certainly incomplete due to the significant differences between the antigenic surface of VHDV, defined by the MAb, and that of EBHSV. A good example is given by the activity of \textit{in vivo} neutralising MAb which is restricted to VHDV (Table I), indicating that the structures implicated in infection/protection processes are different (Ronsholt, personal communication). Moreover, the percentage of VHDV-specific MAb produced against the purified VHDV was quite high, and the cross-reactive MAb were mostly directed against buried epitopes. The same evidence is provided by the hare anti-EBHSV convalescent serum, which reacts significatively less with the non-degraded form of VHDV; this result is also confirmed by sero-epidemiological studies, described later. Rabbit anti-VHDV serum confirms, if somewhat less clearly, the antigenic differences shown by MAb and the convalescent hare serum. This may partly owe to the use of a hyperimmune serum, considerably more “avid” and cross-reactive than a convalescent serum, or to the difficulty in using purified EBHSV in which the degree of proteolytic degradation and the consequent antigenic modifications have been established.

Although limited data are available on \textit{in vivo} cross-protection, the antigenic differences found between VHDV and EBHSV lead to a preliminary classification of these viruses into separate serotypes. The antigenic identity of the two proteolytically degraded viral forms, resulting in “nude” viruses, suggests that the different antigenic areas are specifically located on the spikes radiating from the intact VHD and the EBHS viruses (Figs. 2C and 3B).

**VIRAL IDENTIFICATION**

**Sampling**

Because the liver contains the highest viral titre detected in HA and ELISA (16, 36, 61), it is the organ most usually submitted for the diagnosis of VHD and EBHS. During experimental reproductions, values of the viral concentration within the liver, expressed in Lethal Doses\(_{50}\) (LD\(_{50}\)), ranged from \(10^3\) LD\(_{50}\) to \(10^6\) LD\(_{50}\) (Mortarino, personal communication; 22, 53, 58). The amount of virus present in the various parts is directly proportional to vascularisation (67), probably because of the intense viraemia occurring during the pathogenic evolution of the disease. For this reason, the spleen and the serum become quite rich in virus, and can therefore serve as alternative diagnostic material.

Regardless of the diagnostic method used (HA, ELISA or negative staining EM), the samples are treated in the same manner: an organ fragment is mechanically homogenised in phosphate-buffered saline solution (PBS pH 7.2) at 5-20% w/v and clarified by centrifugation at 3-8,000 rpm for 5-15 min. At this stage the supernatants can be directly examined by HA or ELISA (1, 11, 20, 58). If the sample is to be observed in EM, it is advisable to perform a second centrifugation at 10-12,000 rpm for 5 to 15 min, before the final ultracentrifugation. The virus seems to be quite stable at room temperature, provided that degradation has not already taken place; even so, it is preferable that all steps be carried out at 4°C (13).
Viral isolation

As no satisfactory growth conditions and sensitive cell substrates have yet been identified (13, 16, 25, 28, 33, 47, 52, 68), in vitro isolation of the two viruses cannot be employed as a virological diagnostic method. Attempts on more than 40 primary and continuous cell cultures have failed to support viral replication, although some workers have reported defective or incomplete replication (1, 5, 6, 55). Work is in progress on the possible replication of VHDV on rabbit kidney cell strain (FRK 855) (16), but to date viral isolation in vivo by experimental reproduction of VHD and EBHS retains paramount importance. Its application as a routine diagnostic method is impractical, although for samples which are unusual (HA-negative) or not clearly positive, the biological trial is still desirable. Viral isolation in vivo is necessary to obtain the large quantities of viral antigen needed for laboratory studies of the aetiological agents, preparation of diagnostic reagents and production of an inactivated tissue-derived vaccine (6, 11, 22, 27, 44, 49, 58, 63, 64).

All attempts to infect routine laboratory animals or chick embryos having failed (4, 52, 64), natural hosts are the only species in which the diseases can be reproduced.

The full susceptibility of the animals involved is necessary for the success of experimental trials. As demonstrated by valid laboratory tests (7, 11, 49, 63), this susceptibility depends both on the age of the animal (preferably more than three months) and on the absence of specific antibodies, even at low titres. Owing to the widespread presence of the two viruses, to the lack of information on their viability in the environment and their transmission routes, special care must be taken for a correct execution of the diagnostic trial. The premises in which the experimental reproduction is conducted must be thoroughly cleaned and disinfected, and appropriate precautions should be taken to avoid accidental contaminations during the course of the trial.

More reports exist of the experimental reproduction of VHD than of EBHS. VHD can be reproduced by using filtered (0.2-0.45 µ) and antibiotated organ suspensions, preferably from the liver, inoculated through the most usual routes (intramuscular, intravenous or endonasal). The evolution and the outcome of the trial depend principally on the viral titre contained in the organ suspension. When the disease is clinically evident, the symptoms and post-mortem lesions are similar to those described in the natural form. A rise in body temperature is registered between the 18th-24th h p.i. (69) while, in 95% of the cases, death occurs between the 24th h and the 72nd h p.i., but may still occur until the 6th day p.i. If the disease persists and the evolution becomes chronic, the predominant symptom is jaundice (6, 7, 40). Animals which overcome the disease show only a transient hyperthermia, depression and anorexia, but present an imponent seroconversion, already detectable 5-6 days p.i. (11).

There are few indications on the experimental reproduction of EBHS (Capucci and Scicluna, personal observation; 14, 17); its occasional diagnostic application is even more limited by the expenses involved and the difficulties in finding seronegative animals and keeping them in experimental units.
Electron microscopy

Nearly all workers who have studied VHD have also described its morphology (4, 11, 16, 23, 26, 49, 54, 63, 70). Although some differences have been noticed, all agree that it is an uncoated virus of approximately 32-35 nm in diameter, with a range of 28-42 nm and an icosahedral profile composed of 32 capsomeres. In the diagnostic sample, a high percentage of viral particles present an electron-dense core approximately 23-25 nm in diameter, delineated by a rim from which radiate ten short, regularly distributed peripheral projections (Fig. 2A). These projections most probably correspond to the ten, more external, cylindrical capsomeres (16). The most significant aspect of the degraded viral form is the complete loss of its external portion: the virions become smaller and perfectly hexagonal, with only the capsid rim visible (Fig. 2C) (Lavazza, personal observation). The characteristic cup-like depressions, typical of the Caliciviridae family, are visible only in the purified form (Fig. 2B). The IEM method, also described by Frescura et al. (20) and Smid et al. (63), has been frequently applied by the authors in their laboratory; this method employs a rabbit hyperimmune serum which is incubated with the diagnostic sample for 1 h at 37°C before ultracentrifugation in a Beckman Airfuge at 82,000 rpm for 15 min. The immunological reaction induces the clumping in aggregates of viral particles, easily identified in EM. Comparing the results obtained from about 1,000 samples, examined in parallel with other diagnostic methods, the IEM technique has proved, with respect to sensitivity and specificity, superior to HA and nearly equal to ELISA (8). Its major limitations are the time and cost involved for each sample; IEM should therefore be employed only for doubtful cases after analysis with more rapid and equally reliable tests.

The morphological characteristics of EBHSV and its similarities to VHDV have already been mentioned. EM examination has allowed the identification of the viral aetiology of EBHS (24, 33); for a long time, this method represented the only diagnostic test available for the disease. Valid results have been obtained, especially when performing the IEM procedure with convalescent anti-EBHSV serum and hyperimmune anti-VHD serum (35). In a recent investigation, IEM has proved to be more sensitive than the heterologous VHDV ELISA, and nearly equal to the ELISA utilising MAb (35).

Haemagglutination test

The HA was the first test used for the laboratory diagnosis of VHD. Liu et al. (37) referred to the haemagglutinating property of the aetiological agent towards human erythrocytes type "O" when discussing the first outbreaks. The method, described in detail by Pu et al. (56), has been the most frequently performed diagnostic procedure, having been used first by Chinese (7, 13, 65, 68) and later by European researchers (1, 22, 38, 48, 61). The HA test is based on the capability of VHDV strongly to agglutinate human red blood cells with titres ranging from $10 \times 2^{12}$ to $10 \times 2^{18}$. Haemagglutination is less evident, or null, when erythrocytes of other species are used (6, 20, 37, 56, 68). The test is usually carried out in micromethod. The optimal pH ranges from 6 to 7.2 and the erythrocytes are suspended in saline buffered solution at a concentration of 0.5-1%. No significant differences in titres are observed when incubating the plates at temperatures ranging from 4°C to 37°C for 20 min to 2 h. The virus is spontaneously eluted following 12 h incubation at 37°C. The HA activity remains stable after treating the virus with ether, chloroform
or heating at 50°C for 60 min or at 56°C for 15 min. Cold storage of organs, even for long periods, does not significantly reduce the HA titre; however, repeated freezing and thawing is deleterious. Besides the liver, the spleen, lungs and kidneys also present HA titres with low values. The HA activity of the heart, lymphnodes and muscles is modest or hardly detectable (16, 36).

Although the HA test has been widely applied, its low sensitivity and specificity, combined with difficult standardisation and the need of a constant supply of human haemoderivates, are all factors limiting its use.

Another important issue concerns the reliability of HA. Investigations conducted on approximately 1,000 samples, examined in parallel with IEM, ELISA and HA, demonstrated 8% false positive samples in HA, a situation which probably correlates to the presence of other haemagglutinating agents (i.e. *Pasteurella* and parvovirus). Another 9%, justified by a major sensitivity of the ELISA and confirmed by IEM, were considered as HA false negatives; 4% of these samples contained a low antigen concentration, while in the other 5%, the presence of a degraded form of the virus was responsible for the negative HA result (8).

Apart from being continuously called into question, the HA activity of EBHSV has also been considered as a characteristic which differentiates the two viruses. Hare organs rarely gave a significant titre when the same HA protocol employed for the VHDV was applied (Ronsholt, personal communication; 18, 35). Recently, however, it was possible to verify the presence of HA activity in EBHSV positive organs, with titres ranging from $10^4$ to $10^12$, applying the technique indicated by Dr Gavier (personal communication). In this case, all steps were carried out at 4°C and the liver extract was treated with an equal volume of chloroform. The supernatant obtained from the centrifugation of the samples (approximately 5,000 rpm for 10 min) was successively put into contact with a 0.5% suspension of human red blood cells type “O” at a pH level not higher than 6.5, and the plates were incubated on ice. The sensitivity of the HA reaction to EBHS, even when applying this protocol, remained inferior to that obtained for VHD. Of the total samples diagnosed positive for EBHSV by IEM and ELISA, only about 50% presented an HA activity. At least in part, this was due to the degradation of the examined virus or to its constantly lower concentrations in comparison with those found for VHDV (35).

**ELISA detection of VHD antigen**

Various ELISA reactions, all based on the sandwich method, were set up as an alternative to the HA. They differed from each other both in the enzymatic system and in the type of immunological reagents used as catcher and tracer. In all cases, a rabbit hyperimmune anti-VHD serum was directly adsorbed on the solid phase while the tracers chosen were different; Ronsholt (reviewed in 47) used a hamster hyperimmune serum raised against a purified virus, while Haas (reviewed in 47; 50) employed IgG purified from a second hyperimmune serum from a rabbit other than the one used to sensitise the solid phase. Immunological reagents of different origin were used to diminish the chance of aspecific reactions; these may occur when use is made of a single serum containing antibodies directed against other aetiological agents which may be present in the sample. In their laboratory (47) the authors employed, as both catcher and tracer, the IgG anti-VHD purified from the same hyperimmune serum and had no problem with aspecific reactions. Results from more than 1,000 diagnostic samples confirmed the reliability of the ELISA reaction obtained
by the authors (8). Regardless of the different approaches taken, ELISA has proved significantly more sensitive and specific than HA. In fact, ELISA rescues about 10% positive samples which, because of the previously described proteolytic degradation of the virus, react negatively in HA.

**ELISA detection of EBHS antigen**

The correlation studies conducted by the authors in their laboratory indicated that the ELISA used for the diagnosis of VHD was also applicable to EBHS, though with certain limitations (35). The percentage of false negatives, for example, was as high as 45%, as evinced by IEM, and a clear threshold of positivity was difficult to establish. In fact, the samples reacting positively were those with the highest viral concentrations and/or those which had undergone a relevant proteolytic degradation, thus exposing cross-reactive determinants. An ELISA sandwich, based on a hare anti-EBHSV serum which became available later, was also tested by the authors. The use of a homologous serum allowed to score as EBHSV-positive several hare samples which had previously been negative or doubtful in the VHDV ELISA test. Even if the catcher and the tracer of the reaction again derived from the same serum, no false positives were noticed; moreover, the specificity of the reaction could be confirmed by testing a greater number of samples. The authors do not use the EBHSV homologous serum ELISA test routinely and prefer the available MAb for this purpose. The application of EBHSV homologous serum to a serological method is described in another chapter of this issue.

**Antigen detection using MAb**

As stated earlier, approximately 50 MAb were produced towards the VHDV. This was done, under the supervision of Dr Brocchi, in the MAb production laboratory of the Institute where the authors work. Of the total number of MAb produced, only 20% were cross-reactive with EBHSV (MAb CR); the majority of these were directed towards buried epitopes. While the MAb CR selected by the authors for their studies could not confer protection from infection, the MAb 1H8 and 3H2, chosen from the VHDV-specific group (MAb RS) proved, to varying degrees, capable of *in vivo* protection. Furthermore, the high efficiency of MAb CR 6D6, 6G2 and 3H6 in reactions with those assembling and/or degrading viral subunits constantly present, in significant quantities, at the replication site of the virus, have proved relevant for diagnostic purposes. Besides being effective in diagnosing "haemorrhagic disease", the combined use of the monoclonal and polyclonal antibodies in ELISA also provides a differential diagnostic method capable of distinguishing VHDV from EBHSV.

At present, rabbit and hare samples are both examined in the ELISA sandwich, with the polyclonal anti-VHDV serum as catcher and two pools of MAb, directly conjugated to horseradish peroxidase (HRP), as tracers. The first pool, which assures the diagnosis of "haemorrhagic disease", is made up of cross-reactive MAb 3H6, 6F9, 6D6 and 6G2 (pool CR); the second, which discriminates between the two antigenically different viruses, is composed of MAb 1H8 and 3H2 (pool RS). The sensitivity of the MAb-based ELISA is equal to that of IEM; thanks especially to the characteristic reactivity of the MAb CR, results are superior if samples contain viral subunits largely prevailing on virions.
Since the summer of 1989, all rabbit and hare samples processed by the authors in their laboratory have been assayed according to the antigenic criteria described in this chapter. The results obtained from the above investigation show that the virus present in the rabbit samples always reacts positively with MAb CR and, if not proteolytically degraded, also with the MAb RS. The virus present in the hare samples, however, reacts with the MAb CR but never with the MAb RS. After demonstrating the presence of two correlated caliciviruses, antigenically distinct and easily distinguishable, the diagnostic results indicate that VHD and EBHS should be considered as two different diseases because each is caused by its own aetiological agent.

SEROLOGY

Antibody detection of VHDV

Owing to the haemagglutination property of the virus, HI was the first reaction used for the detection of anti-VHD antibodies (56). All protocols use human type "O" erythrocytes at 0.5-1% and four to eight HA units of antigen; they also state the necessity of pretreating the sera. Sera are inactivated by heat or by kaolin treatment and/or by pre-adsorption to the type "O" red blood cells; while this improves the specificity of HI, it renders the method time-consuming and limits the number of samples which can be tested (21, 49, 61).

To avoid such drawbacks, many laboratories have established a diagnostic procedure based on ELISA. Various protocols are available which can be subdivided into two groups: ELISA competition and indirect ELISA reactions. The competition ELISA was set up by the authors (47, 62) and by Drs Haas (reviewed in 47) and Ronsholt (reviewed in 47) in their respective laboratories. What distinguishes them is the choice of the step at which competition takes place. According to Drs Haas and Ronsholt, the competition reaction starts after the absorption of the virus by the antiserum which coats the solid phase. A preset dilution of enzyme conjugated IgG anti-VHDV is added to the diluted sample sera. After the enzymatic reaction has taken place, the presence of specific antibodies in the sera is detected by the drop in absorbance with respect to the value of the negative control.

In the method followed by the authors, however, competition for the virus took place during the first part of the reaction. The sera, after having been directly diluted on the precoated plate, were incubated with a prefixed concentration of VHD antigen (stored at 20°C in 50% glycerol). After incubation, the amount of specific antibodies present in the sera was then indirectly quantified by binding of HRP conjugated rabbit IgG anti-VHDV. The serum titre was expressed as the dilution, reducing by 50% the absorbance value of the negative control. The serum was considered technically negative if the value of the 1/10 dilution was no more than 20% of the value of the negative control reaction. Rabbits showing such values, employed in experimental reproductions and vaccine control, were susceptible to the disease and mortality was registered in approximately 80% of the cases.
The indirect ELISA has been devised independently by Frescura et al. (21), Rodák et al. (58) and Schirrmieier et al. (61). The assayed sera are incubated on a plate precoated with purified VHDV; after incubation, enzyme conjugated immunoglobulin anti-rabbit IgG are added. The titre of the serum corresponds to the highest dilution giving an absorbance value which is still considered positive. These authors have also demonstrated that it is possible to test a single serum dilution (1:100) to obtain a semiquantitative estimation (21, 58) or to calculate its titre through a reference standard curve (21). By comparing the indirect and competition ELISA assays, the authors have observed that the preparation of the reagents foresees the application of a purified antigen for the indirect reaction, while crude liver extract works well in the competition reaction. On the other hand, the indirect ELISA employs an enzyme conjugated immunoglobulin anti-rabbit IgG commercially available, while the competition reaction requires a “home-made” enzyme conjugated IgG anti-VHD (31). In view of their application on a large scale, both ELISA methods allow the positivity of a sample to be established by testing just one dilution; the indirect reaction, however, permits semiquantitative estimation of the titre within a wider range.

In terms of the capacity to reveal the minimum quantity of specific antibodies present, sensitivity is similar in the two reactions. The tenfold higher positive threshold level of the indirect ELISA is due to the different mechanisms which are proper to each reaction. With regard to specificity, the indirect method is more susceptible to failure because the reaction is unable to distinguish nonspecific antibodies bound to the solid phase. The presence of nonspecific antibodies, especially of the IgM class, may constitute a problem at low serum dilutions. Moreover, the direct adsorption of a small round virus on the solid phase induces a significant change in the viral structure, resulting in the presentation of internal determinants and, therefore, reducing the ability to discriminate between correlated viruses (43). The serological test for foot and mouth disease virus (FMDV) is a well-known example of this situation; all attempts to apply the indirect ELISA have failed because it is incapable of distinguishing specific titres from the different serotypes. For this reason, the competition reaction is decidedly preferable when specificity is the main concern.

In conclusion, the diagnostic application of HI is limited, and the only advantages of the ELISA are the less sophisticated and more easily available reagents employed. For all the other aspects, such as standardisation, rapidity, specificity and sensitivity, the ELISA is the test of choice. This is also the general consensus of the participants in the European Society for Veterinary Virology Meeting on VHD, held in Lyons in December 1989 (47). Other laboratories, however, report less definitive conclusions (21, 58).

Sero-epidemiological results

The results of the sero-epidemiological survey, together with experimental trials and vaccine controls, provide a fairly complete serological picture of VHD. Table II summarises the titres, in relation to the anamnestic data, obtained by the authors in their laboratory. In the case of sera from animals in which no episodes of VHD have been observed, two situations have been identified:

- those relating to animals whose sera were totally negative;
- those relating to animals with titres ranging from average to low (1:10-1:200).
The latter situation was constantly observed in the rabbits kept by the authors in their main experimental unit. After the authors had purposely introduced ten seronegative rabbits, eight of them seroconverted after 30 to 40 days without presenting clinical signs associated with the disease. Reporting on the sera of animals on farms where the disease had not previously been notified, Rodák et al. (58) observed approximately 20% positivity; the percentage rose to approximately 80% in sera of laboratory rabbits collected between 1975 and 1983. The same authors also observed that mortality in such animals was as little as 5%, a further proof of the specificity and protective activity of the titres. As indicated both in the VHD meeting held in Lyons (47) and by Rodák et al. (58), such titres suggest the natural presence of a nonpathogenic virus closely related, and probably antecedent, to VHDV. The vaccinated animals had titres ranging primarily from 1:100 to 1:400 and sometimes reaching 1:1000; this clearly depended on the vaccination schedule and the time of sampling. Convalescent or experimentally hyperimmunised rabbits presented higher titres, ranging from 1:1000 to 1:16000.

Antibody detection of EBHSV

Neither published articles nor personal communications by other authors are available on the subject of antibody detection in EBHSV. In their laboratory the authors initially used the same VHDV ELISA, employing the EBHSV antigen; later, they introduced the ELISA based on convalescent hare sera, as described in the “Virology” section. Both types (VHDV and EBHSV ELISA) have been used for the analysis of field sera; although the results obtained for each system were comparable, the authors chose the homologous EBHSV-specific reaction. The above results are further confirmed by testing EBHSV antigens from distinct outbreaks with sera collected from various geographical areas where no significant differences were noticed in the titres obtained. The antigen used in the routine test was an EBHSV, partially purified and concentrated from a hare liver by pelleting on a sucrose cushion, stored at −20°C in 50% glycerol.

The serological results obtained refer to samples collected from wild hares captured during hunting controls in various areas of Northern Italy, hares reared in captivity and hares imported from other countries (35). The titres were similar to those obtained for the VHD serology and can be briefly summarised as follows:

1) Negative titres, or titres below 1:10, were observed in animals from a limited number of farms and only in one case in wild hares from a restricted geographical area.

2) The most frequent titres, and those of most hares imported from other countries, ranged from 1:40 to 1:320.

### Table II

**Schematic results of the sero-epidemiological survey**

<table>
<thead>
<tr>
<th>Animals</th>
<th>ELISA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Neg./1:200</td>
</tr>
<tr>
<td>Convalescent</td>
<td>1:1000/1:16000</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>1:20/1:1000</td>
</tr>
</tbody>
</table>

The same authors also observed that mortality in such animals was as little as 5%, a further proof of the specificity and protective activity of the titres. As indicated both in the VHD meeting held in Lyons (47) and by Rodák et al. (58), such titres suggest the natural presence of a nonpathogenic virus closely related, and probably antecedent, to VHDV. The vaccinated animals had titres ranging primarily from 1:100 to 1:400 and sometimes reaching 1:1000; this clearly depended on the vaccination schedule and the time of sampling. Convalescent or experimentally hyperimmunised rabbits presented higher titres, ranging from 1:1000 to 1:16000.

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1) Negative titres, or titres below 1:10, were observed in animals from a limited number of farms and only in one case in wild hares from a restricted geographical area.

2) The most frequent titres, and those of most hares imported from other countries, ranged from 1:40 to 1:320.
3) Titres from areas in which cases of EBHS were confirmed by virological diagnosis ranged mostly between 1:600 and 1:5000, occasionally reaching values of 1:40000.

4) Similar titres were observed in hares reared in captivity and in which the disease had occurred about a month before (35). The nearly complete absence of negative titres in hares, apart those observed in captive animals reared in a closed environment, demonstrates the wide diffusion of EBHS. Although the diffusion of the disease has been confirmed by the virological findings, the possible presence of other correlated viruses, as suggested for VHDV, should not be ignored.

Serological correlations between VHDV and EBHSV

Studies on the serological correlations between the two viruses have been conducted; they rely mainly on large numbers of hare sera collected over a vast geographical area. The titres of the hare sera against EBHSV were constantly higher than those obtained against the VHDV, and the ratio between the two sets of titres, titre EBHSV/titre VHDV, depends on the value of the first group. When the titres are low, between 1:40 and 1:160, the ratio is 4 to 16, while for titres considered as average (i.e. 1:320), it varies between 8 to 64, reaching for the highest titres values of 16 to 516. Slightly lower ratio values were registered in a parallel study conducted on convalescent rabbit sera. These results are a further confirmation of the significant antigenic difference, previously demonstrated by the virological results (35, 62), which exists between VHDV and EBHSV.

OTHER DIAGNOSTIC PROCEDURES

The techniques mentioned below have only been briefly described in literature and have been used only on a limited number of occasions. In some cases, especially those relating to the techniques described by Chinese authors, the original papers were not available; information, therefore, had to be obtained from abstracts published in the Veterinary Bulletin. These techniques include the staphylococcal protein A (SPA) ELISA (66), latex agglutination test (Gregg, personal communication), solid phase immuno electron microscopy (SPIEM) (41) and immunoGold (42), all used for the detection of the VHD antigen. The agar gel immuno diffusion test (AGID) has been applied to VHD serology (15, 49, 58).

Owing to its rapidity and the relative ease with which it is carried out, direct immunofluorescence (IF) has been proposed as a valid routine diagnostic test. The method has been described by Nowotny et al. (48) and by Rodák et al. (57), while Dr De Simone (personal communication) has obtained positive results in this laboratory. The reagents used were polyclonal sera directly conjugated to fluorescein isothiocyanate (FITC), or MAb subsequently detected by FITC conjugated immunoglobulin anti-mouse IgG. The immunoperoxidase test (IP) has been used in pathological studies of the disease (Gelmetti, personal communication; 8, 39, 40) and has been reported by Rodák et al. (57) as a valid diagnostic aid if used on thin liver sections. Again, polyclonal sera have been used on histological sections from rabbits (Gelmetti, personal communication) and hares (39, 41), and MAb have been used on tissue sections (Gelmetti, personal communication), liver smears of rabbits (57) and liver sections of hares (39).
CONCLUSIONS

Although attempts at viral isolation on cellular substrates have been unsuccessful, the routine virological diagnosis of the clinically evident forms of VHD and EBHS is relatively simple.

Both the HA and IEM tests and, most especially, ELISA, allow a rapid and reliable identification of VHDV. In view of the difficulties encountered by the HA test in the diagnosis of EBHSV, ELISA is essential; if necessary, the IEM examination can be used as support. The introduction of VHDV-specific MAb has allowed a differential diagnostic method to be set up, one whose results are easy to interpret in relation to the degree of the antigenic diversity identified, at serotype level, between the two viruses.

The ELISA-based methods are preferable to other methods for the serological diagnosis of the two diseases. The sero-epidemiological results so far gathered indicate the presence of VHDV specific antibodies in apparently healthy animals. No valid explanations have as yet been found, apart from the hypothetical existence of nonpathogenic virus, antigenically correlated.

Besides having a direct diagnostic value, the laboratory results also acquire an epidemiological importance. This is supported both by the virological and serological findings which confirm the diseases as two distinct pathologies.

Future improvements in the diagnosis of VHD and EBHS are expected from knowledge yet to be acquired on the aetiological agents, pathogenetic mechanisms and epidemiological behaviour of the two diseases. Such studies will also enhance the parallel development of the technologies of monoclonal antibodies and molecular biology.

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DIAGNOSTIC DE LA MALADIE HÉMORRAGIQUE VIRALE DU LAPIN ET DU SYNDROME DU LIÈVRE BRUN EUROPÉEN. — L. Capucci, M.T. Scicluna et A. Lavazza.

Résumé: Les méthodes de diagnostic pour la maladie hémorragique virale du lapin (VHD) et le syndrome du lièvre brun européen (EBHS) ont progressivement été mises au point au cours des dernières années. Les recherches menées par les auteurs ont démontré que le virus de l’EBHS est un calicivirus, tout comme celui de la VHD du lapin. Le degré de corrélation entre les deux virus est une question essentielle, tant pour la compréhension de leur biologie que pour l’interprétation des résultats de diagnostic. Après avoir exposé les similitudes et les différences existant entre la VHD et l’EBHS, les auteurs présentent les tout derniers résultats concernant la corrélation antigénique entre les deux virus. Compte tenu de l’absence de procédés d’isolement de ces virus en culture, les
méthodes de diagnostic commentées par les auteurs sont : pour la détection du
virus, le test d’hémagglutination (HA), l’immuno-électromicroscopie (IEM) ou
la technique ELISA (titrage immuno-enzymatique utilisant un antigène absorbé)
et, pour la détection des anticorps, l’épreuve d’inhibition de l’hémagglutination
(HI) et la technique ELISA. Les principaux obstacles, au diagnostic de l’EBHS
en particulier, sont passés en revue ; ils portent sur les modifications d’ordre
morphologique, structurel et antigénique, dues à une dégradation protéolytique.
Les auteurs présentent également une méthode de diagnostic différentiel pour
les deux maladies, basée sur la technique ELISA utilisant les anticorps
monoclonaux. Leur conclusion, tirée de l’analyse épidémiologique des données
virologiques et sérologiques, est que la VHD du lapin et l’EBHS doivent être
considérés comme deux maladies distinctes, chacune d’elles étant provoquée
par un agent spécifique.

MOTS-CLÉS : Anticorps monoclonaux - Calicivirus - Diagnostic - Maladie des
lagomorphes - Maladie hémorragique virale du lapin - Syndrome du lièvre brun
européen - Virus VHD - Virus EBHS.

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DIAGNÓSTICO DE LA ENFERMEDAD HEMORRÁGICA VIRAL DEL CONEJO Y DEL
SÍNDROME DE LA LIEBRE PARDA EUROPEA. – L. Capucci, M.T. Scicluna y
A. Lavazza.

Resumen: El desarrollo de los métodos de diagnóstico de la enfermedad
hemorrágica viral del conejo (VHD) y del síndrome de la liebre parda europea
(EBHS) ha conocido en los últimos años un regular progreso. Las investigaciones
realizadas por los autores demuestran que el virus del EBHS es, al igual que
el de la VHD, un calicivirus. El grado de correlación entre los dos virus es de
primer importancia, tanto para entender su biología, como para poder
interpretar los resultados de los diagnósticos. Los autores proceden a una
discusión de las similitudes y diferencias existentes entre la VHD y el EBHS,
para luego presentar los últimos resultados de correlación antigénica entre los
dos virus. En vista de la ausencia de procedimientos para aislar cualquiera de
los dos virus por cultivo, los métodos de diagnóstico que los autores comentan
son: para la detección del virus, la prueba de hemaglutinación (HA), la inmuno-
electromicroscopía, y la técnica ELISA; para la detección de anticuerpos, la
prueba de inhibición de hemaglutinación (HI) y la técnica ELISA. Los autores
describen los principales obstáculos al diagnóstico del EBHS en particular; estos
se manifiestan por unas modificaciones morfológicas, estructurales y antigénicas
causadas por una degradación proteolítica. Se propone luego un método de
diagnóstico diferencial para las dos enfermedades, basada en la técnica ELISA
utilizando los anticuerpos monoclonales. La conclusión final de los autores,
resultante del análisis epidemiológico de los datos virológicos y serológicos, es
que la VHD del conejo y el EBHS han de ser considerados como dos
enfermedades distintas, cada una causada por un agente etiológico específico.

PALABRAS CLAVE: Anticuerpos monoclonales - Calicivirus - Diagnóstico
- Enfermedad de los lagomorfos - Enfermedad hemorrágica viral del conejo
- Síndrome de la liebre parda europea - Virus EBHS - Virus VHD.
REFERENCES


