RABBIT HAEMORRHAGIC DISEASE

SUMMARY

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of the European rabbit (Oryctolagus cuniculus), caused by a calicivirus (genus Lagovirus). A similar disease, [caused by a different calicivirus and] termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus). Its aetiological agent is a different species of lagovirus [calicivirus], antigenically related to the RHD virus (RHDV). RHD is characterised by high morbidity and high mortality (70–90%), and spreads very rapidly by direct and indirect transmission. Infection can occur by nasal, conjunctival or oral routes. Transmission of RHD is facilitated by the high stability of the virus in the environment. The incubation period varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. The clinical manifestations have been described mainly in the acute infection and are nervous and respiratory signs, apathy and anorexia. Clear and specific lesions, both gross and microscopic, are present. There is primary liver necrosis and a massive disseminated intravascular coagulopathy in all organs and tissues. The most severe lesions are in the liver, trachea and lungs. Petechiae are evident in almost all organs and are accompanied by poor blood coagulation. In rabbits younger than 4–5 weeks, the RHDV infection course is subclinical.

Identification of the agent: The liver, spleen and blood of rabbits that died of acute RHD contain a very high concentration of virus. Direct diagnosis is therefore easy and several tests can be used. However, as no sensitive cell substrates are established in-vitro isolation cannot be used. As a consequence, the only available biological assay is reproduction of the disease in sensitive rabbits. The main laboratory tests used are RNA amplification (reverse-transcription polymerase chain reaction [RT-PCR]) and sandwich enzyme-linked immunosorbent assay (ELISA) based on the use of monoclonal antibodies (MAbs). As RHDV haemagglutinates human Group O red blood cells very efficiently, the haemagglutination (HA) test can also be used. False-negative results could arise in the few rabbits that die from the chronic form of RHD (i.e. 4–8 days post-infection). In these rabbits both an anti-RHDV IgM and virus-like particle (VLPs), made by the inner shell of RHDV, heavily interfere with HA and ELISAs. Moreover, using highly sensitive PCR in blood, intestinal contents or faeces from healthy rabbits suspected to be RHD convalescent or RHD re-infected, some non-pathogenic RHDV-related caliciviruses were isolated and partially characterised; importantly these RHDV-related viruses seem quite widespread in both commercial and wild rabbit populations. [The liver contains the highest viral titre and is the most suitable organ for viral identification. As no satisfactory growth conditions or sensitive cell substrates have been established, in-vitro isolation cannot be employed. The haemagglutination test using human Group O red blood cells was the first test applied for routine laboratory diagnosis of RHD. However, other tests (negative-staining electron microscopy, sandwich enzyme-linked immunosorbent assay [ELISA], immunohistological staining, polymerase chain reaction and Western blot) have shown a higher level of specificity and sensitivity.]

Serological tests: The level of RHDV-specific antibodies in the serum is a direct indication of the state of protection of rabbits from RHD. In addition, determining the levels for each class of immunoglobulins (IgM, IgA and IgG) helps in distinguishing the first RHDV infection from re-infection or vaccination. Several types of ELISAs were developed and are currently used as a replacement for the haemagglutination inhibition (HI) test initially used. The total level of RHDV-Ig is semi-quantified using three main ELISAs: a) with the virus adsorbed on the solid phase; b) with the virus and the serum both in liquid phase (competition ELISA), c) with the antigen (virus or subunit) linked to the solid phase throughout MAbs. The different degree of exposition of the internal viral epitopes obtained in these ELISAs helps to distinguish the Ig induced by RHDV from those induced by the non-pathogenic related viruses. [Characterisation and titration of specific antibodies arising from natural infection or from immunisation are performed using the haemagglutination inhibition test or an indirect or
A. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and [اصابة] fatal disease of wild and domestic European rabbits (Oryctolagus cuniculus) older than 2 months. RHD is caused by a calicivirus (genus Lagovirus, family Caliciviridae), a non-enveloped small round RNA virus with only one major capsid protein (VP60) (Capucci et al., 1990; Meyers et al., 1991; Ohlinger et al., 1990). The genus Lagovirus also includes the European brown hare syndrome virus (EBHSV), the causative agent of a disease of brown hare (Lepus europaeus) termed EBHS, very similar to RHD. Despite their high genetic relation (VP60 nucleotide similarity of 70%), RHDV and EBHSV are two distinct viral species (Lavazza et al., 1996; Wirblich et al., 1994). There is only one serotype of RHDV and it consists of two subtypes: RHDV (wild type) and RHDVα (Capucci et al., 1998; Schirmayer et al., 1999).

RHD was first reported in 1984 in China (People’s Rep. of) (Lui et al., 1984) and 2 years later in Europe. To date RHD has been reported in over 40 countries in Asia, Africa, Americas, Europe and Oceania and is endemic in most parts of the world (McIntosh et al., 2007). The RHDV subtype has been identified in Europe since 1996–97 (Capucci et al., 1998; Schirmayer et al., 1999) and is replacing the original RHDV in several parts of the world. However, considering the RHDV genetic sequences deposited at the NCBI databank, the presence of RHDVa in China (People’s Rep. of) dates back to 1985.

RHD is characterised by high morbidity and a mortality rate that usually ranges between 70 and 90%. The incubation period varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. In the few rabbits that survive (5–10%), a specific and relevant IgM response appears within 3 days immediately followed by an IgA and by an IgG response 2–3 days later (Barbieri et al., 2007). Whether this is the consequence of a slow viral clearance mechanism. In spite of this prompt humoral response, 5–10% of infected rabbits die between 4 and 8 days post-infection from a chronic form of RHD. In the liver and spleen of these rabbits, an RHD virus-like particle (VLP) has been detected instead of RHDV (Barbieri et al., 1997; Capucci et al., 1991; Granzow et al., 1996). This VLP is characterised by the lack of the outer shell on the viral capsid made up by the half C-terminal portion of the VP60. As a consequence, this VLP is negative in the haemagglutination (HA) test as well as with most of the anti-RHDV monoclonal antibodies (MAbs) (Capucci et al., 1996a). In the blood and faeces of convalescent rabbits, as well as in rabbits infected with RHDV but already protected by specific antibodies previously acquired (i.e. vaccinated or survivors of infection), the viral RNA is detected using polymerase chain reaction (PCR) for 4–6 weeks post-infection (Gall et al., 2007; Gall & Schirmeyer, 2006). Whether this is the consequence of a slow viral clearance or of a real and prolonged virus replication (persistence) is still to be established.

As a result of RHD serology testing (Capucci et al., 1991; 1997; Collins et al., 1995; Cooke et al., 2000), several rabbit caliciviruses related to RHDV but non-pathogenic have been isolated and partially characterised in Europe and Oceania (Capucci et al., 1996b; 1997; Forrester et al., 2002; Moss et al., 2002; Strive et al., 2009; White et al., 2004). These caliciviruses induce a serological response that may interfere with and complicate RHD serological diagnosis (Capucci et al., 1991; Cooke et al., 2000; Marchandau et al., 2005; Nagesha et al., 2000; Robinson et al., 2002).

The causative agent of RHD is a calicivirus that is 32–35 nm in diameter and has a single major capsid polypeptide (60 kDa), a positively stranded RNA genome of 7437 kb and a sub-genomic RNA of 2.2 kb (8, 28, 29, 33). The RHD virus (RHDV) capsid protein (VP60) folds in two distinct domains held together by a hinge region: the N-terminal 200–250 residues constitute the inner domain and the C-terminal residues beyond 250–250 constitute the protruding domain. In the overall picture of the capsid, these domains form the inner shell and the outer shell, respectively, which is characterised by arch-like structures. This structure...
also correlates with the antigenic characteristics of RHDV, and the main antigenic determinants are located on the C-terminal end of the VP60 (3, 4, 35, 41).

Since 1991, a second type of virus particle was identified as the main component in approximately 5% of the RHDV-positive specimens, i.e., those taken from rabbits showing a protracted course of the disease (7). The characteristics of this particle are: i) it has an smooth surface and a diameter smaller than RHDV; ii) its protein is of 28–30 kDa; iii) it is reactive with RHD-convalescent rabbit sera and with those anti-RHDV monoclonal antibodies (MAbs) reacting against the N-terminal end of the RHDV VP60; and iv) it is haemagglutination (HA) negative. This smaller viral particle only corresponds to the inner shell of RHDV, and two hypotheses have been proposed to explain its origin. Granzow et al. (19) assumed that it arose from a truncated RHDV genome or defective expression. However, Barbier et al. (1) observed the following: i) a strict correlation between the higher prevalence of smooth RHDV-(s-RHDV)-particles in the organs and the appearance of specific anti-RHDV IgM at 3–4 days post-infection; ii) the presence of large amounts of s-RHDV only in the liver and spleen and not in the bloodstream, as occurs during the viræmic phase of acute RHD; and iii) the finding of fragments of the VP60 having different molecular weights (41–30 kDa) during transition from RHDV to s-RHDV. They concluded therefore that the genesis of the s-RHDV particle is due to a degradative process that is probably the consequence of the physiological clearance of the RHDV-igM immuno-complex formed in large amounts at the beginning of the humoral response. Apart from its origin, the identification of this second particle in the liver of a rabbit can be considered to be a marker of the subacute/chronic form of RHD that usually evolves between 4 and 8 days post-infection and is followed either by the death of the rabbit or, more often, by its recovery (1).

Most known RHD-viral isolates appear to belong to one serotype. The complete sequence of geographically different RHD strains has been reported. Comparison reveals close overall homology in terms of genome sequence with few or no predicted changes in amino acid composition (differences between 2% and 5%). Nevertheless, isolates that exhibit temperature-dependent differences in haemagglutinating characteristics (2) have been described, and more recently a consistent genetic and antigenic RHDV variant has been identified simultaneously in Italy (3) and Germany (35). This RHDV variant, named RHDVa, presents amino acid changes in the surface-exposed E region (aa 344–434) that contains the main antigenic epitopes of calicivirus, three times higher than in all previously sequenced RHDV isolates. A group of related MAbs that protected against infection by RHDV were negative when tested by enzyme-linked immunosorbent assay (ELISA) against RHDVs antigen. However, rabbits experimentally vaccinated with the currently available RHDV vaccine were protected against challenge with RHDVs, even if with a lower efficiency (3, 35).

An epidemiological study carried out to compare the rate of spread of RHDV and RHDVs in Italy during the past few years (24) has shown that RHDV is present in most parts of Italy and that it is rapidly replacing the RHDV ‘classical’ strain. Outside Italy, RHDV was identified almost contemporaneously in Germany, but it also caused the first outbreaks of RHD in the USA during 1983. It has since been found showing that pathogenic strains may usually be present in wild European rabbit populations, because high antibody levels have been detected in areas where RHD had never been recorded or suspected (27).

Antibodies against RHD were detected in sera collected in Europe between 1975 and 1987, showing that RHDV-like viruses were already present, but had simply not been detected before the first signs of the disease. More recent serological data suggest that non-pathogenic strains may usually be present in wild European rabbit populations, because high antibody levels have been detected in areas where RHD had never been recorded or suspected (27).

A study carried out in Britain showed that mRNA particles related to RHDV were present in sera collected since 1965, confirming that RHDV-like viruses were present in Europe a long time before the first evidence of RHD (30). However, according to the authors’ data (30, 40), RHDV causes a highly prevalent persistent infection in susceptible rabbits in the absence of associated mortality and, since the responsible viral strain could not be phylogenetically distinguished from known pathogenic isolates, they suggest that “many — perhaps most — strains of RHDV may be propagated through both ‘pathogenic’ and ‘non-pathogenic’ modes of behaviour.”

RHDV is very stable and persists in the environment; the viral infectivity is not reduced by treatment with ether or chloroform and trypsin, by exposure to pH 3.0, or by heating to 50°C for 1 hour. The virus survives at least 225 days in an organ suspension kept at 4°C, at least 105 days in the dried state on cloth at room temperature, and at least 2 days at 60°C, both in organ suspension and in the dried state (36). Recent work suggests that RHDV in rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental conditions is viable for a period of less than 1 month (22). RHDV also retains its infectivity at low temperatures, and remains quite stable during freezing and thawing. RHDV is inactivated by 1% sodium hydroxide and by other agents (e.g. bleach) that cause destruction of the viral proteins by increasing the pH to >12. Treatment with 1.0-1.4% formaldehyde or 0.2-0.5% beta-propiolactone at 4°C inactivates the virus but does not reduce its immunogenicity and is therefore indicated for the production of vaccines.

The European rabbit (Oryctolagus cuniculus) is the only species known to be affected by RHD. No other lagomorphs, such as the Volcano rabbit of Mexico (Romerolagus diazi), the black-tailed jackrabbit (Lepus californicus) and the cottontail (Sylvilagus
florianus) of North America, have been shown to be susceptible (20). Inoculation of tissue suspensions from infected rabbits into 28 different vertebrate species other than rabbits failed to produce disease and no replication of the virus was detected by reverse transcription-polymerase chain reaction (RT-PCR) (19). A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus), but its aetiologic agent, EBHSV, also a calicivirus, is distinct from RHDV while still sharing some antigenic similarity. Alignment of the RNA sequences of the EBHSV and RHDV genomes shows 71% nucleotide identity, and amino acid alignment shows 78% identity and 87% similarity (41). Cross infection does not occur by experimental infection of rabbits with EBHSV and hares with RHDV (23). Recent studies aimed at finding the susceptibility of cottontail rabbits to EBHSV revealed a diffuse seroprevalence of the virus in a wild population of cottontail rabbits and the possibility of inducing clinical disease and mortality in a low number of experimentally infected cottontails (Lavazza, unpublished data).

RHD is characterised by high morbidity and a mortality rate between 40% and 90%. Infection occurs in rabbits of all ages, but clinical disease is observed only in adults and animals older than 40–50 days. The pathogenic mechanism of resistance in young animals is still unclear (7). A difference in the cellular inflammatory response of the liver following RHDV infection of susceptible adult and resistant young rabbits has been observed. The persistence of increased liver transaminases following RHDV infection in young rabbits may be an indicator of subacute or chronic infection, which could have implications for virus transmission.(13, 14).

While the clinical evolution of the disease can be peracute, acute, subacute or chronic, clinical manifestations have been described mainly in the acute infection, as there are usually no clinical signs of disease in the peracute form, and the subacute form is characterised by similar but milder signs. The incubation period varies between 1 and 3 days; death may occur 12–36 hours after the onset of fever (>40°C). During an outbreak, a limited number of rabbits (5–10%) may show a chronic or subclinical form of the disease. These animals often die 1 or 2 weeks later, probably due to a liver dysfunction. Gross pathological lesions are variable and may be subtle. Liver necrosis and splenomegaly are the primary lesions. Gross findings include a pale swollen friable liver, enlarged spleen and the presence of clotted blood in blood vessels caused by disseminated intravascular coagulopathy (DIC). Such massive coagulopathy is usually the cause of haemorrhages in a variety of organs and sudden death (20, 39). In subacute and chronic disease, an icteric discoloration of the ears, conjunctiva and subcutis is clearly evident.

The clinical signs and the gross and microscopic lesions observed in hares affected by EBHS are very similar to those described in rabbits with RHD. At necropsy, oedema and congestion of tracheal mucosa with foamy haemorrhagic contents, liver degeneration, enlargement of the spleen and generalised jaundice are the principal findings (7). The disease in hares lasts slightly longer and causes a lower mortality rate (around 50%) than RHD in rabbits; the peak of mortality in experimentally infected hares is commonly observed between 60 and 90 hours post-infection.

### B. Diagnostic Techniques

#### 1. Identification of the agent

The liver contains the highest viral titre (from 10^3 LD_{50} [50% lethal dose] to 10^{6.5} LD_{50}/ml of 10% homogenate) and is the organ of choice for viral identification for both RHDV and EBHSV. The amount of virus present in other parts of the body is directly proportional to vascularisation; thus spleen and serum may serve as alternative diagnostic materials, albeit suboptimal. Higher levels of VLPs [viral particles] have been reported in the spleen than in the liver of animals that died from a subacute/chronic form of RHD (Barbieri et al, 1997; Capucci et al, 1991; Granzow et al, 1996). The initial treatment of the diagnostic samples is almost identical irrespective of the diagnostic method to be applied, with the exception of immunostaining techniques. An organ fragment is mechanically homogenised in 5–20% (w/v) phosphate buffered saline solution (PBS), pH 7.2–7.4, filtered through cheesecloth and clarified by centrifugation at 5000 g for 15 minutes. At this stage, the supernatant can be directly examined by the HA test or enzyme-linked immunosorbent assay (ELISA). If the sample is to be observed by electron microscopy (EM), it is advisable to perform a second centrifugation at 12,000 g for 15 minutes, before the final ultracentrifugation. For detection by PCR, viral RNA from the samples may be also directly extracted from tissues [or from the unclarified homogenate].

#### a[s]) Enzyme-linked immunosorbent assay

Virus detection by ELISA relies on a ‘sandwich’ technique and several variations of this have been described. One procedure uses the reagents, solutions, times and temperature that are used in the competitive ELISA (C-ELISA) for serology (see Section B.2.b), except that the Tween 20 concentration is twofold (0.1% [w/v]). The microplate used should be of high adsorption capability (e.g. Nunc Maxisorp immunoplate). The liver homogenate is a 10% (w/v) suspension in standard PBS; 50 µl/well is the standard volume to use in each step. The ELISA buffer used for all steps is PBS with 1% yeast extract (or bovine serum albumin [BSA]), and 0.1% Tween 20, pH 7.4. All incubation steps are for 50–60 minutes at 37°C with gentle agitation. After all steps three washes of 3–5 minutes must be performed using PBS with 0.05% Tween 20. A positive and negative RHD rabbit liver homogenate must be used as controls. The horseradish peroxidase (HRPO) conjugate could be purified IgG from a specific polyclonal serum or MAbs (see Section B.2.b). Anti-RHDV MAbs have been produced in several laboratories and can be used instead of rabbit polyclonal sera. [More recently] MAbs recognising specific epitopes expressed only by the RHDV a variant were also produced (Capucci, pers. data).
To better characterise the antigenicity of the RHD isolates by sandwich ELISA, it is advisable to test each sample in four replicates, and then to use four different HRPO conjugates, i.e. two MAbs recognising the same antigenic determinant present on the virus surface and expressed alternatively by the ‘classical’ strain or by the RHDVα variant, a polyclonal hyperimmune anti-RHDV serum (which could identify potential ‘new variant’ or correlated calicivirus, such as EBHSV) and a pool of MAbS recognising internal epitopes that can detect smooth, degraded VLPs [RHDV particles] as well as EBHSV. An alternative antigen-capture ELISA has been described using a sheep anti-RHDV as the capture antibody and a MAb for detection of RHDV (Collins et al., 2001).

**Test procedure (example)**

For steps that are not specifically indicated see the procedure of the C-ELISA for serology (Section B.2.b).

i) Coat the plate with anti-RHDV hyperimmune serum and the negative RHDV serum. The latter serves as control for nonspecific reactions (false-positive samples). For each sample, four wells must be sensitised with the positive serum and four wells with the negative one.

ii) Dilute the liver extract to 1/5 and 1/30 (two replicates for each dilution) in ELISA buffer (see above), directly in the wells of the plate (e.g. add 45 µl of the buffer into all the wells of the plate, add 10 µl of the sample to the first two wells and then, after rocking, transfer 9 µl to the second wells). Treat the controls, both positive and negative, in the same way as the samples.

iii) After incubation and washing (see above), incubate with the HRPO conjugate.

iv) After a last series of washing, add the chromogenic substrate. Orthophenylene-diamine (OPD) can be used as peroxidase substrate for the final development of the reaction. Use 0.15 M citrate phosphate buffer, pH 5.0, with 0.5 mg/ml OPD and 0.02% H₂O₂. The reaction is stopped after 5 minutes by the addition of 50 µl of 1 M H₂SO₄.

v) Absorbance is read at 492 nm. Positive samples are those showing a difference in absorbance >0.3, between the wells coated with RHDV-positive serum and wells coated with the negative serum. Usually, at the dilution 1/30, positive samples taken from rabbits with the classical acute form of RHD give an absorbance value >0.8, while the absorbance value of the negative sample, at the dilution 1/5, ranges from 0.1 to 0.25.

For diagnosis of EBHSV, it is possible to use this RHDV-specific sandwich ELISA, but, due to the high antigenic difference existing between the two viruses, there is a risk of obtaining false-negative results. Therefore, the adoption of an EBHSV-specific sandwich ELISA technique using either a high-titre positive anti-EBHSV hare serum, or cross-reacting RHDV MAbS, instead of rabbit serum, is highly recommended (Capucci et al., 1991).

### Nucleic acid recognition methods

The application of the RT-PCR to the detection of RHDV-specific nucleic acid has been described by several authors (Collins et al., 1995). Owing to the low level of sequence variation among RHDV isolates and the high sensitivity of PCR, reverse transcription (RT)-PCR represents an ideal rapid diagnostic test for RHD. This method is carried out on organ specimens (optimally liver), urine, faeces and sera using different oligonucleotide primers derived from the capsid region of the RHDV genome (N-terminal portion). cDNA obtained from the RT reaction is usually PCR amplified as described by Guittre et al. (Guittre et al., 1995). To reveal the PCR product, the amplified DNA reaction mixture is subjected to electrophoresis on agarose gel. If needed, specificity of the PCR product can be determined by sequencing or by Southern blot and hybridisation with a radioactively labelled internal probe. A similar RT-PCR method has been used to identify the nonpathogenic RCV (Capucci et al., 1998). Several primers, specific for the RHDV RNA polymerase gene and complementary to the VP60 and ORF2 genes, are used and the amplified fragments are subjected to Southern blot analysis. RT-PCR represents an extremely sensitive method for the detection of RHDV, and is 10⁴-fold more sensitive than ELISA (Guittre et al., 1995). It is not strictly necessary for routine diagnosis, but it is more sensitive, convenient and rapid than other tests.

Similarly an RT-PCR for the detection of EBHSV has been applied to the detection and characterisation of EBHSV stains (Le Gall-Reculé et al., 2001). An internally controlled multiplex real-time RT-PCR using TaqMan probes and external standards for absolute RNA quantification has been developed recently as a further diagnostic tool for the detection of RHDV. The test revealed a specificity of 100%, an analytical sensitivity of 10 copies/well and a linearity over a range from 10¹ to 10⁶ copies. The method has been used to quantify RHDV RNA in experimental infection of vaccinated rabbits and in RHD convalescent rabbits (Gall et al., 2007; Gall & Schirrmeier, 2006).

An *in-situ* hybridisation technique using either sense or antisense DNA probes has been developed for investigating the presence of RHDV in tissue samples (Gelmetti et al., 1998). This method is highly
sensitive and can be used for early diagnosis of RHD as it gives positive results as soon as 6–8 hours post-infection. However, it is expensive and difficult to carry out, and thus it is mainly indicated for research studies.

**[b]** Electron microscopy

Negative-staining EM can be performed using the so-called ‘drop method’. A formvar/carbon-coated grid is placed on a drop of organ suspension (prepared as described in Section B.1), and left for 5 minutes. After removing excess fluid with the edge of a torn piece of filter paper, the grid is put to float on a drop of 2% sodium phosphotungstate (NaPT), pH 6.8, for 1.5 minutes. Excess stain is removed and the grid is finally observed at ×25,000 magnification.

Due to the lower sensitivity of the drop method, it is advisable to ultracentrifuge the sample in order to concentrate the viral particles. The pellet obtained after ultracentrifugation (at least 100,000 g for 30 minutes or, alternatively, using Beckman Airfuge at 21 psi for 5 minutes) is resuspended in PBS or distilled water, put on to a grid for a few minutes, and then stained as described. RHD virions are visible as uncoated particles, 32–35 nm in diameter, presenting an inner shell (25–27 nm in diameter), delineated by a rim from which radiate ten short regularly distributed peripheral projections. Smooth (s-RHDV) particles are identified by the complete loss of external portions, becoming perfectly hexagonal and smaller, with only the capsid rim visible (Barbieri et al., 1997; Capucci et al., 1991; Granzow et al., 1996[a]).

For diagnostic purposes and especially when other methods give doubtful results, the best EM method is an immuno-EM technique (IEM). This method uses either a hyperimmune anti-RHDV serum, obtained from rabbit or other species, or specific MAbs, which are incubated with an equal volume of the sample for 1 hour at 37°C before ultracentrifugation. The immunological reaction induces the clumping of the viral particles into aggregates that are quickly and easily identified by EM. Immunogold methods can also be applied to better identify virions and viral proteins.

EBHSV can also be identified in diagnostic samples by EM examination. In addition, the IEM method using convalescent anti-EBHSV serum or specific anti-EBHS MAbs can be used to identify EBHSV. By using antisera that is specific for EBHSV and RHDV, it is possible to differentiate between the two viruses.

**[a]** Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (Lui et al., 1984[a]). It should be performed with human Group O red blood cells (RBCs), freshly collected, stored overnight in Alsever’s solution, and washed in 0.85% PBS at pH 6.5 (range 6–7.2). HA is less evident or non-existent when RBCs of other species are used. Washed RBCs are suspended at 0.75% in PBS. A twofold dilution of the clarified supernatant of a 10% tissue homogenate of liver or spleen is incubated with an equal volume of washed RBCs in a sealed round-bottom microtitre plate at, preferably, 4°C. After 1 hour (range from 20 minutes to 2 hours) of incubation, agglutination at an end-point dilution of >1/160 is considered to be positive. Lower titres should be regarded as suspicious, and should be checked using other methods. Around 10% of samples found to be positive by ELISA or EM give negative results in HA (HA false-negative). Some RHD isolates may exhibit temperature-dependent differences in haemagglutinating characteristics (Capucci et al., 1996[a]) and could show HA activity only when the test is performed at 4°C. Nevertheless, the HA false negativity is mainly detected in organs of rabbits showing a subacute/chronic form of the disease and it depends on the characteristics of the VL Ps [smooth, truncated s-RHDV particles].

Hare organs rarely give a significant titre when the RHDV HA protocol is used. To demonstrate HA activity in organs from EBHSV-infected rabbits, a modified procedure should be adopted: all steps are carried out at 4°C, the organ suspension is treated with an equal volume of chloroform, and RBCs are used at a pH not higher than 6.5 (Capucci et al., 1991). Even using this method, only about 50% of the samples give positive results. This is because the disease of hares is often subacute or chronic and therefore the virus has the antigenic and structural characteristics typical of the VL Ps [s-RHDV] particles (Capucci et al., 1991).

Because of the practical difficulty of obtaining and keeping and the risk from working with human Group O blood cells, and because of the difficulty of obtaining consistent results, this test has been replaced by the virus-detection ELISA.

**[a]** Immunostaining

Tissue fixed in 10% buffered formalin and embedded in paraffin can be immunostained using an avidin–biotin complex (ABC) peroxidase method. The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin for 1 minute and rinsed in tap water. They are then put in a methanol bath containing 3% H2O2 and washed in PBS three times for 5 minutes each. To limit background interference caused by nonspecific antibody binding, the samples are incubated with normal rabbit serum for 1 hour at room temperature prior to the addition of biotin. The slides are incubated overnight in a humid chamber at
room temperature with biotinylated rabbit anti-RHDV serum or MAbs, are washed as before and incubated again for 30 minutes at 37°C with an ABC peroxidase. The slides are then washed three times. Amino-ethyl-carbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted (Stoerckle-Berger et al., 1992[22]).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic cells in the liver, mainly in the perportal areas, are characteristic and specific. Positive staining of macrophages and Kupffer’s cells is also observed, as well as hepatocellular reactions. Positive reactions can also be detected in the macrophages of the lungs, spleen and lymph nodes, and in renal mesangial cells (Stoerckle-Berger et al., 1992[22]).

Tissue cryosections fixed in methanol can be directly immunostained by incubation for 1 hour with fluorescein-conjugated rabbit anti-RHDV serum or MAbs. Specific fluorescence can be detected in the liver, spleen, and renal glomeruli.

**f[a]) Western blotting**

When other tests such as HA or ELISA give doubtful results (low positivity) or the samples are suspected of containing s-RHDV particles, western blotting analysis is useful for determining the final diagnosis.

Homogenates are prepared as described previously, and virus particles are further concentrated (tenfold) by ultracentrifugation (100,000 g for 90 minutes) through a 20% (w/w) sucrose cushion.

Both the supernatant and the pellet can be examined to detect, respectively, the RHDV 6S subunits (Capucci et al., 1995) and the denatured VP60 structural protein of RHDV or its proteolytic fragments, which can range in size from 50 to 28 kDa. A positive and negative control samples should be used on each occasion.

RHDV proteins could be detected with polyclonal antibodies or MAbs. If MAbs are used, they should recognise continuous epitopes. RHDV-specific MAbs recognising internal or buried epitopes could be used also to detect EBHSV. Rabbit anti-RHDV hyperimmune sera are less efficient than MAbs at recognising the same band patterns (Capucci et al., 1996b).

Sample proteins are denatured for 2 minutes at 100°C in the presence of 60 mM Tris, pH 6.8, 2% sodium dodecyl sulphate (SDS), 2% beta-mercaptoethanol, and 5% glycerol, separated on 10% SDS/PAGE (polyacrylamide gel electrophoresis), and then transferred by electroblotting to nitrocellulose or PVDF (polyvinylidiene flouride) membranes, in 25 mM Tris, 192 mM glycine pH 8.3 and 20% (v/v) methanol at 1.5 Å for 60 minutes with cooling or at 0.15 Å overnight. After transfer the membranes are saturated for 30–60 minutes in blocking buffer or PBS, pH 7.4 containing 2% bovine serum albumin (BSA), and subsequently incubated for 2 hours at room temperature with the appropriate serum dilution in PBS, pH 7.4, and 1% BSA. The filters are washed thoroughly with PBS and incubated for 1 hour at room temperature with anti-species alkaline phosphatase-labelled immunoglobulins at a dilution predetermined by titration. Finally, the filters are again washed and the chromogenic substrate (5-bromo-4-chloro-3-indolyphosphate nitro blue tetrazolium) is added.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights of, respectively, 60 kDa (the single structural protein of RHDV) or 41–28 kDa (the fragments of the VP60 associated with the transition from RHDV to s-RHDV), when examining the pellet, and 6 kDa (the subunits) when examining the supernatant.

Western blot analysis can also be used to identify EBHSV. The test procedure is identical. The pattern of protein bands, detected using either an anti-EBHSV polyclonal serum or cross-reacting anti-RHDV MAbs, is similar. However, the percentage of samples showing viral degradation is higher and therefore several fragments of lower molecular weight, originating from the VP60 structural protein, are often observed.

**g) Rabbit inoculation**

As no efficient [satisfactory growth conditions] in-vitro replication system has been established for [isolation of] RHDV and [or] EBHSV, cell culture isolation cannot be included among the diagnostic methods. [Inoculation of tissue suspensions from infected rabbits failed to produce disease in 28 different vertebrate species other than rabbits; no replication of the virus was detected by reverse transcription polymerase chain reaction (RT-PCR) in the inoculated animals (18).] Rabbit inoculation therefore remains the only way of isolating, propagating and titrating the infectivity of the RHDV. However, experimental infection of rabbits is not a practical method for the routine diagnosis of RHD and should be considered only when all other methods give inconclusive results. When this occurs, the rabbits involved must be fully susceptible to the virus, i.e. they should be over 2 months old and have no RHDV antibodies (see serological methods) but could be useful for the testing of samples that give equivocal test results (e.g. HA negative/ELISA positive) or for the initial diagnosis of the disease in countries where RHD is not known to exist. Propagation of RHDV in rabbits is also useful for large-scale production of viral antigen for diagnostic
reagents or to produce inactivated RHDV vaccines. To perform successful experimental trials, the rabbits involved must be fully susceptible to the virus, i.e. they should be over 40–50 days and have no specific antibodies, even at low titrers. RHD can be reproduced by using filtered and antibiotic-treated liver suspensions, inoculated either by the intramuscular, intravenous or oro-nasal route. When the disease is clinically evident, the signs and post-mortem lesions are similar to those described after natural infection. A rise in body temperature is registered between 18 and 24 hours post-infection, followed, in around 70–90% of cases, by death between Robinson et al., 2002[24] and 72 hours post-infection. A few individuals may survive until 6–8 days after infection. Animals that overcome the disease show only a transient hyperthermia, depression and anorexia, but present a striking seroconversion that can be detected easily 3–4 days post-infection.

2. Serological tests

Infection by RHDV can be diagnosed through detection of a specific antibody response. As the humoral response has great importance in protecting animals from RHD, determination of the specific antibody titre after vaccination or in convalescent animals is predictive of the ability of rabbits to resist RHDV infection.

Three basic techniques are applied for the serological diagnosis of RHDV: haemagglutination inhibition (HI) (Lui et al., 1984[26]), indirect ELISA (i-ELISA) and C-ELISA (Capucci et al., 1991). Each of these methods has advantages and disadvantages. With respect to the availability of reagents and the technical complexity of carrying out the test, HI is the most convenient method, followed by the I-ELISA and C-ELISA, respectively. On the other hand, both ELISAs are quicker and easier than HI, particularly when a large number of samples are tested. The specificity of the C-ELISA is markedly higher than those achieved with the other two methods (Capucci et al., 1991). An alternative C-ELISA method has been described (Collins et al., 1995). For improved serological interpretation and for correctly classifying the immunological status of rabbits, a combination of ELISA techniques that distinguish IgA, IgM and IgG antibody responses is also available.

Some other additional tests (Capucci, unpublished data; Cooke et al., 2000) could be used for particular investigations and when a higher level of sensitivity is needed to detect antibodies in non-target species or antibodies induced by cross-reacting RHDV-like agents (see Section A. Introduction).

They are:

- **I-ELISA**: the antigen is linked to the solid phase by a RHDV-specific MAb (1H8). It has a slightly higher sensitivity than C-ELISA, making possible measurement of highly cross-reactive antibodies and it can detect antibodies with low avidity.

- **Solid-phase ELISA (SP-ELISA)**: the purified antigen is directly adsorbed to the solid phase and because of virus deformation, internal epitopes are exposed. Therefore it detects a wider spectrum of antibodies and has high sensitivity and low specificity. *For these reasons it can also be used for EBHSV serology.*

- **Sandwich ELISA to detect IgM and IgG in liver or spleen samples already examined with the virological test**: such a test is particularly useful in those animals that die from the ‘chronic’ form of the disease, when detection of the virus may be difficult. In this case, a high level of RHDV-specific IgM and a low level, if any, of IgG are the unambiguous markers of positivity for RHD.

a) **Haemagglutination inhibition**

**Antigen**: The antigen is prepared using infected rabbit liver collected freshly at death. The liver is homogenised in 10% (w/v) PBS, pH 6.4, and clarified by two consecutive low speed centrifugations (500 g for 20 minutes and 6000 g for 30 minutes). The supernatant, drawn from the tube so as to avoid the superficial lipid layer, is filtered through a 0.22 µm pore size mesh, titrated by HA, and divided into aliquots, which are stored at –70°C.

**Serum samples**: Before testing, sera are inactivated by incubation at 56°C for 30 minutes. The sera are then treated with 25% kaolin (serum final dilution: 1/10) at 25°C for 20 minutes and centrifuged. This is followed by a second kaolin treatment, also at 25°C for 20 minutes, this time with 1/10 volume of approximately 50% packed human Group O RBCs. These are freshly collected, stored overnight in Alsever’s solution and washed in 0.85% PBS, pH 6.5. The sera are clarified by centrifugation.

**Test procedure**

i) Dispense 50 µl of serum into the first well of a round-bottom microtitre plate and make double dilutions into wells 2–8 using PBS with 0.05% BSA.

ii) Add 25 µl of RHDV antigen containing 8 HA units to each well and incubate the plate at 25°C for 30–60 minutes.

iii) Add 25 µl of human Group O RBCs at 2–3% concentration to each well and allow to settle at 25°C for 30–60 minutes.
iv) Titrate the antigen with each test to ensure that 8 HA/25 µl were used, and include positive and negative serum controls.

The serum titre is the end-point dilution showing inhibition of HA. The positive threshold of serum titres is correlated to the titre of the negative control sera; it usually is in the range 1/20–1/80.

Because of the practical difficulty of obtaining, keeping and the risk from working with human Group O blood cells, and because of the difficulty in obtaining consistent results, this test is being superseded by the serological or antibody-detection ELISA.

b) Competitive enzyme-linked immunosorbent assay

Antigen: An international standard strain is not yet available; however, as only one serotype has been identified so far world-wide, reliable results can be obtained by different laboratories each using their own standard virus. Even the antibodies induced by the identified RHDV variants are recognised by the standard method described here. In addition, the test can also easily detect antibodies originating from infection of rabbits with the nonpathogenic RCV, because of its high genetic correlation with RHDV (Capucci et al., 1996b; 1997). The antigen can be prepared as described previously for HI (Section B.2.a), taking care to store it at -20°C in the presence of glycerol at 50% (v/v) to prevent freezing. If necessary, the virus can be inactivated before the addition of glycerol, using 1.0% binary ethylenimine (BEI) at 33°C for 24 hours. Antigen must be pretitrated in ELISA and then used as the limiting reagent: i.e. the dilution that corresponds to 60–70% of the plateau height (absorbance value at 492 nm in the range 1.1–1.3).

Anti-RHDV serum: specific polyclonal sera with high anti-RHDV titre can be obtained in different ways. Two possible and currently used methods are as follows:

i) Rabbits are infected with a RHDV-positive 10% liver extract diluted 1/100 in PBS to obtain convalescent sera (21–25 days) containing a high level of anti-RHDV IgG. Due to the high mortality rate associated with RHDV, it is necessary to infect at least 10–15 seronegative rabbits or to infect rabbits that are only partially protected (e.g. 4–8 rabbits infected from 3 to 7 days post-vaccination). Rabbits that survive the infection must be bled 21–25 days post-infection to obtain the convalescent sera. Alternatively, convalescent rabbits can be re-infected 3–4 months post-infection and bled 10–15 days later to obtain RHDV hyperimmune sera.

ii) RHDV is purified from the livers of experimentally infected rabbits that died from an acute form of the disease (between 28 and 40 hours post-infection), using one of the methods that has been published (Capucci et al., 1990; 1991; 1995; Meyers et al., 1991; Ohlinger et al., 1990[18, 33]). Then the purified RHDV can be used to immunise sheep or goats according to classical protocols using oil adjuvants. The same procedure can also be used to inoculate rabbits if the purified virus is inactivated before inoculation.

Anti-RHDV MAbs may be used instead of rabbit polyclonal sera. Purification of rabbit IgG and conjugation to HRPO can be done following the standard protocols. The conjugated antibody is titred in a sandwich ELISA in the presence and absence of RHDV antigen (negative rabbit liver). It is then used at the highest dilution showing maximum (plateau high) absorbance (if the serum had a good anti-RHDV titre, the value of the HRPO conjugate should range from 1/1000 to 1/3000).

Control sera: negative serum is taken from rabbits fully susceptible to RHDV infection. Positive serum is either a convalescent serum diluted 1/100 in a negative serum or a serum taken from a vaccinated animal.

m) Test procedure (example)

i) The rabbit anti-RHDV serum diluted to a predetermined titre, e.g. 1/5000 in 0.05 M carbonate/bicarbonate buffer, pH 9.6, should be adsorbed to an ELISA microplate of high adsorption capability (e.g. Nunc Maxisorb Immunoplate) at 4°C overnight.

ii) Wash the plate three times for 3–5 minutes each time, in PBS, pH 7.4, with 0.05% Tween 20 (PBST). When the plates are not immediately used, they can be stored, closed in a plastic bag, for 1 month at -20°C.

iii) Distribute 25 µl/well PBST with 1% yeast extract (PBSTY) or 1% BSA (PBST-BSA) to all the wells needed on the plate (see below). Add 7 µl of the first serum sample to the first two wells (A1 and B1), 7 µl of the second serum to the second two wells (C1 and D1), and continue with the third (E1 and F1) and the fourth (G1 and H1) sera, thus completing the first column. If qualitative data (positive/negative) are needed, repeat the operation in the second column with sera samples from 5 to 8, and in the third column with sera samples from 9 to 12, and so on. If the titre of the serum needs to be determined, the serum must be diluted further. Agitate the plate and then use an eight-channel micropipette to transfer...
7 µl from the wells in column 1 to the wells in column 2. This corresponds to a four-fold dilution of the sera. This last operation can be repeated once (titre 1/160), twice (titre 1/640), or four times (titre 1/10,240). Either in the case of testing sera for qualitative data (single dilution), or for getting the final titre (several dilutions), complete each plate leaving 12 wells free for the control sera. Add 7 µl of positive sera to wells G7 and H7, and 7 µl of negative sera to wells G10 and H10, then dilute them once and twice (1/40–1/160).

iv) Add 25 µl/well antigen suspended in PBSTY to all the wells on the plate, at a dilution that is double the decided dilution, as described above in the antigen section (see the first part of this ELISA method description).

v) Incubate the plate at 37°C on a rocking platform for 50–60 minutes.

vi) Wash the plate as described in step ii.

vii) Add 50 µl/well rabbit IgG anti-RHDV conjugated with HRPO at the decided dilution, as described above in the ‘anti-RHDV serum’ section (see the first part of this ELISA test description).

viii) Incubate the plate at 37°C on a rocking platform for 50–60 minutes, and wash as described in step ii adding a fourth wash of 3 minutes duration.

ix) Use 50 µl/well OPD as hydrogen donor under the following conditions: 0.5 mg/ml OPD in 0.15 M phosphate/citrate buffer, pH 5, and 0.02% H2O2. Stop the reaction after 5 minutes by addition of 50 µl/well 1 M H2SO4.

x) Read the plate on a spectrophotometer using a 492 nm filter.

The serum is considered to be negative when the absorbance value of the first dilution (1/10) decreases by less than 15% of the reference value (dilution 1/10 of the negative control serum), while it is positive when the absorbance value decreases by 25% or more. When the absorbance value of the 1/10 dilution decreases by between 15% and 25% of the reference value, the sera is considered to be doubtful.

The serum titre corresponds to the dilution giving an absorbance value equal to 50% (±10) of the average value of the three negative serum dilutions.

A wide range of titres will be found, depending on the origin of the sample. Positive sera range from 1/640 to 1/10,240 in convalescent rabbits, from 1/80 to 1/640 in vaccinated rabbits and from 1/10 to 1/160 in ‘non-pathogenic’ infection. Knowing the origin of the sample allows a choice to be made between testing one or more dilutions. Testing only the first dilution gives a positive or negative result. The titre is established by testing all dilutions, up to the sixth one.

Due to the significant antigenic differences existing between RHDV and EBHSV (Capucci et al., 1990; Stoerckle-Berger et al., 1992), the serological techniques described above, which use RHDV as antigen, are not recommended for the serological diagnosis of EBHS. However, a direct ELISA method could be employed for the detection of positive and negative EBHSV hare sera; in fact, the adsorption of RHDV on to the solid phase of an ELISA microplate exposes cross-reactive antigenic determinants. Alternatively, a specific C-ELISA for EBHSV can be arranged in a similar way, using specific reagent (antigen and antisera) prepared as described above for RHDV.

c) Isotype enzyme-linked immunosorbent assays (isoELISAs)

These isoELISAs enable the detection and titration of isotypes IgA, IgM and IgG (Capucci et al., 1997). Isotype titres are critical for the interpretation of field serology in four main areas: cross-reactive antibodies, natural resilience of young rabbits, maternal antibodies, antibodies in previously infected rabbits (Cooke et al., 2000[12]). In fact in the case of passive antibodies, only IgG are detected; in vaccinated animals, no IgA are detected and in recently infected rabbits, first IgM and then IgA and IgG are detected (Cooke et al., 2000).

To detect RHDV-specific IgG, one RHDV-specific MAb is adsorbed to the Maxisorp plate at a concentration of 2 µg/ml by the method described above for the polyclonal serum in the C-ELISA (see above Section B.2.b, test procedure step i). Virus is added to the plates at a concentration double that used in the C-ELISA and after incubation and washing; sera are added and serially diluted four-fold starting from 1/40. A MAb anti-rabbit IgG HRPO conjugate is used to detect IgG bound to the virus. The final step for the isoELISAs for IgG, IgM and IgA is the addition of OPD and H2SO4 as for the C-ELISA. To detect IgM and IgA isotypes the phases of the ELISA reaction are inverted in order to avoid competition with IgG, which is usually the predominant isotype. MAb anti-rabbit IgM or anti-rabbit IgA is adsorbed to the wells and then the sera are diluted as described above. Incubation with the antigen follows and then HRPO-conjugated MAb is used to detect the RHDV bound to the plate. Sera are considered to be positive if the OD492 (optical density) value at the 1/40 dilution is more than 0.2 OD units (two standard deviations) above the value of the negative serum used as a control. The titre of each serum is taken as the last dilution giving a positive value. Because
isoELISA tests do not follow identical methodology, equivalent titres do not imply that isotypes are present in the same amounts.

C. REQUIREMENTS FOR VACCINES [AND DIAGNOSTIC BIOLOGICALS]

1. Background

a) Rationale and intended use of the product

In countries where RHD is endemic, indirect control of the disease in farmed animals and pet rabbits is achieved by vaccination using the appropriate type of vaccine – one that is prepared from clarified liver suspension of experimentally infected rabbits, and that is subsequently inactivated and adjuvanted. The methods of inactivation (formaldehyde, beta-propiolactone or other substances) and the adjuvants used (incomplete mineral oil or aluminium hydroxide), can vary according to the protocol used by the different manufacturers.

[In the last ten years several studies were carried out on the expression of RHDV capsid protein in Escherichia coli, in vaccinia virus, and in attenuated myxoma virus (MV). Moreover, it has been shown by various authors that a recombinant capsid protein, VP60, expressed in the baculovirus/Sf9 cell expression system, self-assembled into virus-like particles (VLPs) that are structurally and antigenically identical to RHDV virions. While the fusion protein expressed in E. coli is highly insoluble and of low immunogenicity, active immunisation can be achieved with VLPs obtained in the baculovirus system or by using recombinant vaccinia, MV and canarypox, administered either intramuscularly or orally. In particular rabbits vaccinated with recombinant MV expressing the RHDV capsid protein were protected against lethal RHDV and MV challenges. The resulting recombinant virus was also capable of spreading horizontally and promoting protection of contact animals, thus providing the opportunity to immunise a wild rabbit population (38). Similarly, the immunogenicity of VLPs administered by the oral route as an alternative to parenteral immunisation offers an economical and practical way to administer a vaccine for mass immunisation of wild animals.

More recently, the VP60 structural protein has been expressed in transgenic plants, either with a new plm-pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. In both cases the immunisation of rabbits with extracts of Nicotiana clevelandii plants infected with the PPV-NK VP60 chimera and with leaf extracts from potatoes carrying this modified 35S promoter, respectively, induced an efficient immune response that protected animals against a lethal challenge with RHDV. However, at the present time, recombinant vaccines are not yet registered and commercially available.

In France, a vaccine (Dercunimix®, Merial) has recently been commercialised that is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated Myxovirus vaccine, and which can be administered by the intradermal route.]

The usual programme is to administer the inactivated vaccine twice with an interval of at least 2 weeks. Normally, a 1-ml dose is inoculated subcutaneously in the neck region. In those units with no history of disease, where the anamnesis for RHD is negative, it is advisable to vaccinate only the breeding stock; the first injection should be given at 2–3 months of age and booster revaccination is administered after 3–4 weeks. Annual revaccination is strongly recommended to ensure a good level of protection, although experimental data indicate that protection usually lasts for a long time (over 1 year).

Given the short life-cycle (approximately 80 days) of fattening rabbits and their natural resistance to the disease until 35–40 days of age, vaccinating these rabbits is not necessary if the situation on the farm is normal, i.e. good biosecurity measures are applied and there are no outbreaks of the disease in the area.

[The vaccination of meat animals is not necessary if disease has not occurred on the farm.] Following an outbreak of RHD, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, it is strongly recommended to vaccinate meat animals at the age of 40 days, because the incidence of re-infection is very high. Only after several production cycles is it advisable to stop vaccination of meat animals. To verify the persistence of infective RHD inside the unit, a variable number of rabbits, starting with a small sentinel group, should not be vaccinated.

Given that immunity starts after about 7–10 days, vaccination could also be considered a quite effective post-exposure treatment. In some situations in particular it may be included in the emergency strategies applied when RHD occurs on those farms having separate sheds and where good biosecurity measures are regularly applied. Indeed, better results in limiting the diffusion of the disease and reducing economic losses could be obtained by using serotherapy through the parenteral administration of anti-RHDV hyperimmune sera, which produces a rapid, but short-lived, protection against RHDV infection. [Vaccinated animals quickly produce strong immunity against RHDV infection, therefore vaccination is considered to be effective in protecting non-exposed rabbits and its primary use is in rabbits after an outbreak of the disease has been diagnosed; once RHD has been confirmed in some sick or dead rabbits, the remaining healthy animals are immediately vaccinated.

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The administration of immune serum is also effective in producing a rapid, but short-lived, protection against RHDV infection.

Vaccine should be stored at 2–8°C and it should not be frozen, or exposed to bright light or high temperatures.

Seed management

Nowadays it does not result that vaccine programmes against RHD are used for the wild rabbits.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed
   i) Biological characteristics
      At present, RHDV replication can be obtained exclusively in susceptible animals. Therefore, the source of seed virus for the production of inactivated tissue vaccines is infected liver homogenates obtained by serial passages in rabbits that have been inoculated with a partially purified RHD viral suspension. The rabbits used for inoculation are selected from colonies shown to be healthy and susceptible to the disease by periodic serological testing.
   ii) Quality criteria (sterility, purity, freedom from extraneous agents)
      The partially purified RHD viral suspension is obtained by centrifuging the 1/5 liver suspension (w/v) in PBS at 10,000 g for 20 minutes at 4°C. The resulting supernatant is treated with 8% (v/v) polyethylene glycol (PEG 6000) overnight at 4°C. The pellet is resuspended at a dilution of 1/10 in PBS, and subsequently centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant is ultracentrifuged at 80,000 g for 2 hours at 4°C through a 20% cushion of sucrose. The pellet is resuspended in PBS (1/100 of the starting volume).
      This viral suspension is then characterised by negative-stain EM examination, determination of reactivity in ELISA, and capability of HA at room temperature (with slow elution) (HA titre against RBCs of human Group O higher than 1/1280).
      The absence of viable bacteria, fungi or mycoplasma should be determined by using common laboratory bacteriological methods. PCR methods may be used for the detection of extraneous viruses (e.g. Myxoma virus [MV]).
      Seed virus is controlled by direct inoculation into susceptible rabbits followed by evaluation of the clinical signs in the course of the experimental infection. Suitable seed virus should cause the death of 70–80% of the rabbits within 24–72 hours post-inoculation, with the internal organ lesions characteristic of RHD. To validate the test, gross and histopathological examination of all rabbits should be performed to exclude undercurrent diseases.
      Seed virus is titrated before use and should contain at least 10⁵ LD₅₀. It should be stored frozen (−70°C), better with the addition of 1:1 volume of glycerol or freeze-dried.

b) Method of culture
   At present, RHDV replication can be obtained exclusively in susceptible animals. The rabbits used for inoculation are selected from colonies shown to be susceptible to the disease by periodic serological testing. The animals (at least 4 months old) must be kept in strict quarantine on arrival, in a separate area and reared under satisfactory health conditions (see Laboratory animal facilities in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Seed virus propagation and production of vaccine batches relies on the same protocol of experimental infection, involving intramuscular injection of a dose of at least 100 LD₅₀.

c) Validation as a vaccine
   The seed virus used for vaccine production must be shown to be free from other viruses, bacteria, mycoplasma and fungi. Seed virus is controlled by direct inoculation into susceptible rabbits followed by evaluation of the clinical signs in the course of the experimental infection. Suitable seed virus should cause the death of 70–80% of the rabbits within 24–72 hours post-inoculation, with the internal organ lesions characteristic of RHD. To validate the test, gross and histopathological examination of all rabbits should be performed to exclude undercurrent diseases.

b) Method of manufacture
   i) Procedure
      Following inoculation of susceptible rabbits, the liver and spleen of those rabbits that die between 24 and 72 hours post-inoculation are collected. Rabbits that died later must be discarded. The organs are minced in 1/10 (w/v) sterile PBS, pH 7.2–7.4, and the mixture is homogenised for 10 minutes in a blender in a refrigerated environment. The mixture is then treated with 2% chloroform (18 hours at 4°C),
followed by centrifugation at 6000 \(g\) for 1 hour at 4°C. The supernatant is collected by high pressure continuous pumping and is subsequently inactivated. The viral suspension is assayed by HA test and ELISA [see Section C.3.] and, once the number of HA units from the initial titration is known, more sterile PBS is added in sufficient volume to provide, after inactivation and adsorption/addition of the adjuvant, a concentration of 640–1280 HA units/ml in the commercial product. Various agents have proved effective at abolishing viral infectivity. The most frequently used are formaldehyde and beta-propiolactone, which can be used at different concentrations and temperatures, for variable periods of time and also in combination. During inactivation, it is advisable to continuously agitate the fluid. Aluminium hydroxide, Freund’s incomplete adjuvant or another oil emulsion is then incorporated into the vaccine as adjuvant. A preservative, thiomersal (merthiolate), is finally added at a dilution of 1/10,000 (v/v) before distribution into bottles.

**ii) Requirements for substrates and media**

As the virus cannot be grown in vitro, the only requirements are those concerning infected animals. Rabbits must be free from RHDV and myxomatosis virus and should not have anti-RHDV antibodies, including cross-reactive antibodies induced by the non-pathogenic RHDV-related rabbit calicivirus (RCV).

The animals (at least 4 months old) must be kept in strict quarantine on arrival, in a separate area and reared under satisfactory health conditions (see Laboratory animal facilities in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

Seed virus propagation and production of vaccine batches rely on the same protocol of experimental infection, involving intramuscular injection of a dose of at least 100 LD\(_{50}\).

**iii) In process controls**

**Antigen content**

The RHD titre is determined before inactivation by calculating the HA titre, which should be higher than 1/1280, and the ELISA reactivity. Both values are again determined after inactivation and adsorption/addition of the adjuvant. Negative-staining EM confirms the identity of RHD.

**Sterility**

The organs are tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the protocol used for testing master seed virus. PBS solution and aluminium hydroxide gel are sterilised by autoclaving; oil emulsion is sterilised by heating at 160°C for 1 hour.

**Inactivation**

Before incorporation of the adjuvant, the inactivating agent and the inactivation process must be shown to inactivate the vaccine virus under the conditions of manufacture. Thus, a test is carried out on each batch of the bulk harvest as well as on the final product. [Five rabbits are inoculated with a 2 ml dose of the suspension and five unvaccinated rabbits are kept as controls. After 10 days, adequate inactivation and absence of undesirable side-effects are demonstrated by the absence of clinical signs of disease and by similar weight increments in the two groups. At the end of the trials, the animals are slaughtered and liver extracts are tested by HA, ELISA and EM.]

**4. Batch control**

Thirty adult rabbits (>4 months of age) are used in three groups of 10. The first and second group are injected with concentrated antigen and kept under observation for 15 and 7 days, respectively. The second group is humanely killed after 7 days. The third group is injected with the liver of rabbits from the second group and kept under observation for 21 days. The dose of the inoculum, administered parenterally (intramuscular or subcutaneous), is 1 ml of concentrated antigen (PEG precipitation) corresponding to at least 10 doses (HA ≥20480). The observation period is: 10 rabbits for 7 days, 10 rabbits for 15 days and 10 rabbits for 21 days. All the rabbits kept under observation must survive without any clinical signs. The liver should give negative results using the HA test and sandwich ELISA. The rabbits inoculated with antigen should have a positive serological titre (e.g. >1/80 using competitive ELISA) and those injected with livers obtained after the first passage should be serologically negative.

**iv) Final product batch tests**

Sterility, safety and potency tests should be carried out on each batch of final vaccine; tests for duration of immunity should be carried out once using a typical batch of vaccine, and stability tests should be carried out on three batches.

**Sterility/purity**

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Each batch of vaccine must be tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the same protocol recommended for testing master seed virus.

**Safety**

[Ten rabbits should be inoculated by the recommended routes with three times the vaccinal dose. The rabbits are observed for 2 weeks. No abnormal local or systemic reaction should develop.

c) **Potency**

Ten seronegative adult rabbits that are at least 4 months old are vaccinated with one full dose of vaccine given by the recommended route. Two other groups of five animals each are vaccinated with 1/4 and 1/16 of the full dose, respectively. A fourth group of ten unvaccinated rabbits is maintained as controls. All animals are challenged at 4 weeks post-vaccination by intramuscular inoculation of a dose of RHDV containing at least 100 LD$_{50}$ or presenting a HA titre higher than 1/2560. No vaccinated rabbits should show signs of infection, while the mortality rate among control animals should be higher than 70%. The antibody response of each vaccinated animal is then determined with reference to titrated standard antisera; the mean antibody level should not be.

Before proceeding with field trials, the safety of this new vaccine is tested in laboratory studies. The following safety tests in particular should be carried out:

i) The safety of the administration of one dose;

ii) The safety of the administration of an overdose (at least two doses of inactivated vaccine);

iii) The safety of the repeated administration of one dose.

The test is carried out for each approved route of administration. Use at least 10 adults (>4 months of age) that are RHDV antibody free. Observe these animals for 21 days by evaluating the following life parameters: general conditions and reactions, sensory condition, water and food consumption, characteristics of faeces, and local abnormal reactions at the inoculum point. Record the body temperature the day before vaccination, at vaccination, 4 hours after vaccination and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction should occur; the average body temperature increase should not exceed 1°C and no animal should have a temperature rise greater than 2°C. A local reaction lasting less than 21 days may occur. If the vaccine is intended for use in pregnant rabbits, administer the vaccine to not less than 10 pregnant does according to the schedule to be recommended. Prolong the observation period until 1 day after parturition. The does should remain in good health and there should not be abnormal local or systemic reactions. No adverse effects on the pregnancy or on the offspring should be noted.

**Batch potency**

Use susceptible adult rabbits (>4 months old), free from antibodies against RHDV and reared in suitable isolation conditions to ensure absence of contact with RHDV. Ten rabbits are vaccinated with one full dose of vaccine given by the recommended route. Two other groups of five animals each are vaccinated with 1/4 and 1/16 of the full dose, respectively. A fourth group of 10 unvaccinated rabbits is maintained as controls. All animals are challenged not less than 21 days post-vaccination by intramuscular inoculation of a dose of RHDV containing at least 100 LD$_{50}$ or presenting a HA titre higher than 1/2560. Observe the rabbits for a further 21 days. The test is valid if: a) during the period between vaccination and challenge more than 10% of the vaccinated or more than 20% of control rabbits show abnormal clinical signs or die from causes not attributable to the vaccine; b) less than 70% of control rabbits died with typical signs of RHD. The vaccine complies with the test if: a) not less than 90% of vaccinated rabbits show no signs of RHD; b) the mean antibody level of vaccinated animals, is not significantly less than the level recorded in the protection test performed using as vaccine the inactivated seed virus.

**d) Duration of immunity**

The data reported in the literature indicate a long-term duration of immunity induced by a single vaccination (up to 15 months). However, it is advisable to carry out the following test: 20 rabbits vaccinated once are divided into four groups and are serologically tested at monthly intervals over a period of 1 year. Each group is inoculated with virulent RHDV at 3, 6, 9 months or 1 year post-vaccination (see Section C.4.c). Challenge infection should produce increasing seroconversion, which is directly related to the time that has elapsed since vaccination. The absence of clinical signs of disease and mortality supports the efficacy of the vaccine.

**c) Requirements for authorisation**

The tests for safety, potency and sterility of the final product must be performed after bottling and packaging. Thus, it is important that these two last manufacturing steps be performed following standardised good manufacturing procedures. The tests are conducted by removing samples from a statistically determined number of randomly taken multi-dose containers (20, 50 or 100 doses) of vaccine.

i) Safety requirements
**Target and non-target animal safety**

Rabbit is the sole species susceptible to RHDV and in the interest of animal welfare, tests and trials must be held only on target animals. The safety requirements of the final product for rabbits should be verified in field studies on both fattening and breeder rabbits. At least 30 breeder rabbits, ≥4 months of age, and 70 rabbits 30–45 days of age should be used. Breeder rabbits are vaccinated subcutaneously at the back of the neck twice (at an interval of 3 weeks) with one dose. Fattening rabbit are vaccinated either at 30 or 45 days of age. Animals are observed for 4 months from the first vaccination. Unvaccinated animals are kept as controls.

The control of the safety of the vaccine in breeder rabbits is done by evaluating their reproductive performance. The following parameters are considered: local or general reactions; total number of born rabbits and the number of live rabbits born; percentage of mortality at the weaned period; average weight of bunnies at the weaned period; daily consumption of food. The control of the safety of the vaccine in fattened rabbits is done by evaluating their daily health. The following parameters are considered: local or general reactions; individual weight increase from weaning (30 days) and every 15 days; daily consumption of feed; conversion index; mortality during the fattening period. Vaccinated rabbits should not show any changes in their general conditions or abnormal local or systemic reactions for the whole test duration.

The vaccine should not contain any ingredients that are likely to pose a risk for consumers of vaccinated rabbits. However, as the inactivated vaccine contains a mineral oil adjuvant, there is an associated risk that might arise from accidental self injection. Accidental injection can cause intense swelling and severe consequences if expert medical advice is not sought promptly.

**Reversion-to-virulence for attenuated/live vaccines**

Reversion-to-virulence does not occur because it is an inactivated vaccine.

**Environmental consideration**

During the safety and efficacy field trials, interactions with other vaccines (e.g. vaccine against myxomatosis) or pharmaceutical products (medicated feeding-stuffs containing antibiotics against respiratory diseases and bacterial enteritis) should be checked and recorded. No interactions have been reported to date.

The inactivated vaccine does not spread in the environment and, in previous trials, there were no signs of ecotoxicity problems for the viral antigens. The risk of ecotoxicity caused by the use of vaccine is zero because of the nature of the vaccine (inactivated vaccine for parenteral use). The vaccine contains no ingredients likely to pose a risk to the environment. In addition, the vaccine is administered by injection so environmental contamination is unlikely. To achieve the highest standard of safety, in accordance with good hygiene rules, the bottles must be dipped in an antiseptic solution after use.

### ii) Efficacy requirements

The efficacy should be tested in the laboratory with both challenge and serology tests. Forty rabbits (20 vaccinated and 20 unvaccinated), at least 4 months of age, are challenged with virulent virus: all unvaccinated animals must have died within the observation period.

Before the first vaccination, the C-ELISA should confirm, in all rabbits, the total absence of anti-RHDV antibodies or the presence of titres at the lower acceptance limit of the protection titre ≤1/10. Vaccinated animals develop an RHDV protective immunity in a short period of time: in the serum of infected animals, circulating antibodies are present just 3–4 days post-infection (IgM and IgA). whereas in rabbits vaccinated with the inactivated and adjuvanted vaccine, the first antibodies usually appear after 7–10 days (only IgM). IgG appear after approximately 15–20 days. After vaccination there is very low or no IgA production. As it is produced only during infection with the live virus following oro-nasal dissemination, IgA could be considered to be a marker of contact with the field virus. The mucosal immuno-system may also be involved in protection to the disease even if the vaccine is parentally and not orally administered. This is suggested by oral challenge experiments in vaccinated rabbits when IgA but no IgM appear very quickly in the serum. This suggests that B memory cells able to produce IgA are already present at the mucosal level, which is usually the first site of replication of RHDV.

There is a definite correlation between the titre in the C-ELISA and the state of protection from the disease, i.e. rabbits with titres higher than 1/10 for antibodies specifically induced by RHDV did not show any sign of disease when challenged with virulent RHDV. In convalescent rabbits, serological titres could be as high as 1/20480, whereas in vaccinated rabbits they are usually between 1/40 and 1/640 according to the time elapsed since vaccination. Maternal antibodies (IgG only) usually disappear within 30 days of age in young rabbits born to vaccinated healthy does, but they last longer.
(until 45–55 days of age) when rabbits are born to convalescent does, as the passive titres of young are directly related to that of their mothers. This is true of young rabbits from industrial farms that are weaned quite early (25–35 days of age), whereas in young wild rabbits, maternal antibodies can last for 80 days (Forrester et al., 2002). In young rabbits (<35–40 days old), a low level of antibody (1/80–1/320) could also be induced by an active infection not leading to disease, as commonly occurs in animals of this age.

The data reported in the literature indicate the long-term duration of immunity induced by a single vaccination (up to 15 months). At 9–12 months post-vaccination, titres are 2–4 times lower than observed 2–3 weeks after vaccination. The booster effect, in the case of natural infection or re-vaccination, depends on the time elapsed since vaccination, i.e. it is lower 5–7 months post-vaccination and higher in animals vaccinated before that time.

To exactly determine the duration and efficacy of immunity, it is advisable to carry out the following test: 20 rabbits vaccinated once are divided into four groups and are serologically tested at monthly intervals over a period of 1 year. Each group is inoculated with virulent RHDV at 3, 6, 9 months or 1 year post-vaccination. Challenge infection should produce increasing seroconversion, which is directly related to the time that has elapsed since vaccination. The absence of clinical signs of disease and mortality supports the efficacy of the vaccine.

### Stability

Evidence should be provided to show that the vaccine passes the batch potency test at 3 months beyond the suggested shelf life.

### Preservatives

A suitable preservative is normally required for vaccine in multi-dose containers. Its persistence throughout shelf life should be checked.

### Vaccines based on biotechnology

Several studies have been carried out on the expression of RHDV capsid protein in *Escherichia coli*, in vaccinia virus, and in attenuated *Myxoma* virus (MV). Moreover, it has been shown by various authors that a recombinant capsid protein, VP60, expressed in the baculovirus/Sf9 cell expression system, self-assembled into VLPs that are structurally and antigenically identical to RHD virions. While the fusion protein expressed in *E. coli* is highly insoluble and of low immunogenicity, active immunisation can be achieved with VLPs obtained in the baculovirus system or by using recombinant vaccinia MV and canarypox, administered either intramuscularly or orally. In particular, rabbits vaccinated with recombinant MV expressing the RHDV capsid protein were protected against lethal RHDV and MV challenges. The resulting recombinant virus was also capable of spreading horizontally and promoting protection of contact animals, thus providing the opportunity to immunise a wild rabbit population. Similarly, the immunogenicity of VLPs administered by the oral route as an alternative to parenteral immunisation offers an economical and practical way to administer a vaccine for mass immunisation of wild animals.

The VP60 structural protein has also been expressed in transgenic plants, either with a new plum pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. In both cases the immunisation of rabbits with extracts of *Nicotiana clevelandii* plants infected with the PPV-NK VP60 chimera and with leaf extracts from potatoes carrying this modified 35S promoter respectively, induced an efficient immune response that protected animals against a lethal challenge with RHDV. However, at the present time, recombinant vaccines are not yet registered and commercially available.

In France, a vaccine has been commercialised that is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated *Myxoma* virus vaccine, and which can be administered by the intradermal route.

### Precautions

- **When oil-emulsion vaccines are prepared, vaccinators should be warned against the risk and consequences of accidental self-injection, which must be treated urgently as a ‘grease-gun’ injury.**

### Tests on the final product

The tests for safety, potency and stability of the final product must be performed after bottling and packaging. Thus, it is important that these two last manufacturing steps be performed following standardised good manufacturing procedures. The tests are conducted by removing samples from a statistically determined number of randomly taken multidose containers (20 or 100 doses) of vaccine.

### Safety
See Section C.4.b.

b) Potency

See Section C.4.c.

c) Sterility

See Section C.4.a.

REFERENCES


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**NB:** There is an OIE Reference Laboratory for Rabbit haemorrhagic disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).