RABBIT HAEMORRHAGIC DISEASE

SUMMARY

Description and importance of the disease: *Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal hepatitis of Leporids, caused by a calicivirus (*genus* Lagovirus). Up to 2010, all RHD viruses (RHDV) isolated belonged to one of the previously identified six genotypes (G1– G6), among which the G6 is an antigenic subtype (RHDVa). In 2010, an additional RHDV, phylogenetically and antigenically distinct from RHDV, emerged in Europe and was called RHDV2. Another lagovirus, called the European brown hare syndrome virus (EBHSV), causes a similar disease in hares (*Lepus europaeus, L. timidus *and* L. corsicanus).*

RHD is characterised by high morbidity and a mortality of up to 90%. Infection mainly occurs by the oral route. Transmission follows contact with infected rabbits or via indirect contact with mechanical vectors (including insects, birds and humans) or contaminated tools and equipment. The incubation period of RHD varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. The main clinical manifestations of the acute infection are nervous and respiratory signs, dullness and anorexia. In rabbits younger than 4–6 weeks, the RHDV/RHDVa infection course is subclinical, but when the causative agent is RHDV2, clinical signs and mortality are observed even in young animals from 7 to 15 days of age onwards.

Detection and identification of the agent: *The liver and spleen of rabbits that died of acute RHD contain a very high concentration of virus: consequently, several test methods can guarantee a reliable diagnosis. Considering that no sensitive cell substrates have been established* in-vitro*, 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). Specific primers and MAbs should be selected and used to distinguish among different lagoviruses. As RHDVs haemagglutinate human Group O red blood cells, the haemagglutination (HA) test can also be used bearing in mind that HA-negative RHDV variants have also been identified. The detection of RHDV particles in liver homogenates by electron microscopy is also possible. The diagnosis of chronic RHD can be complicated by the presence of high anti-RHDV antibody titres in the samples, causing possible false-negative results in ELISA and especially in HA tests.*

Serological tests: *Humoral immunity is the main defence against RHD and even a low level of specific and homologous anti-RHDV antibodies confers protection from the disease. The best RHD serological methods are based on the competitive ELISA using specific MAbs. These methods also allow RHDV and RHDV2 infection or vaccination to be distinguished in previously uninfected rabbits. In addition, ELISA quantification of RHDV-specific isotype immunoglobulins (IgM, IgA and IgG), helps in distinguishing the first infection from re-infection or vaccination. Classical direct ELISA, which needs purified RHDVs or recombinant virus-like particles (VLPs) to adsorb to the solid phase, shows a high diagnostic sensitivity. However, the exposition of the internal common epitopes shared by different lagoviruses decreases the test's specificity.*

Requirements for vaccines: *Indirect control of the disease is easily achieved by vaccination. Although RHDV capsid proteins have been expressed as recombinant VLPs and are commercially available, most vaccines used are still prepared from the livers of infected rabbits and are inactivated and adjuvanted. Vaccinated animals produce solid protective immunity against RHDV infection and experimental data indicate that protection lasts for a long period (over 1 year). As RHDV and RHDV2 have different antigenic profiles, combined vaccination with both serotypes, or the use of a vaccine homologous to the RHDV or RHDV2 strain identified during the epidemics or the outbreak, is highly advisable.*

A. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal hepatitis of leporids. RHD is caused by a calicivirus (genus *Lagovirus*, family Caliciviriade), a non-enveloped small round RNA virus with only one major capsid protein (VP60) (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. Despite their high genetic relationship (VP60 nucleotide similarity of 70%), RHDV and EBHSV are two distinct viral species (Capucci *et al.,* 1991; Green *et al.,* 2000; Wirblich *et al.,* 1994).

A new classification of the genus *Lagovirus* has been proposed based on the VP60 coding sequences (Le Pendu *et al.,* 2017). In the proposal, not yet approved by the International Committee on the Taxonomy of Viruses (ICTV), all lagoviruses are grouped in a unique virus species (*Lagovirus europaeus*). This could be divided in two genogroups (GI and GII) that can be subdivided into six genotypes (GI.1, GI.2, GI.3, GI.4, GII.1 and GII.2), and further into variants (four for GI.1 and three for GII.1). According to the new genome classification, the genomes of "classical RHDV", including previous genogroups G1–G5, first reported in China (People's Rep. of) in 1984 (Liu *et al.*, 1984), and the subtype RHDVa previously corresponding to genogroup G6, identified in Europe in 1996 (Capucci *et al.*, 1998), are now classified as GI.1 (a–d). The new RHDV-related virus called RHDV2 (or RHDVb), which emerged in France in 2010 (Le Gall-Recule *et al.*, 2013), is now classified as GI.2. The EBHSV genome is classified as GII.1. However, as the official ICTV taxonomy for Caliciviridae family is still that described by Green *et al.* (2000), the current *Lagovirus* classification is used in this chapter.

The European rabbit (*Oryctolagus cuniculus*) is the only susceptible host species for RHDV and RHDVa. Other lagomorph species, including cottontails (*Sylvilagus* spp.), black-tailed jackrabbits (*Lepus californicus*) and volcano rabbits (*Romerolagus diazzi*) are not susceptible. The RHDV2 host spectrum is broader as it can also cause disease in the Sardinian cape hare (*L. capensis* var *mediterraneus*), the Italian hare (*L. corsicanus*), the brown hare (*L. europaeus*), the mountain hare (*L. timidus*) and various hare (*Lepus*) and cottontail (*Sylvilagus*) species in North America, even if with a different degree of susceptibility.

EBHSV commonly causes disease in *Lepus europaeus, L. timidus* and *L. corsicanus,* and occasionally in cottontails (*Sylvilagus floridanus*), but apparently not in *L. granatensis*, *L. castroviejoi* and *L. capensis.*

RHDV has never been reported in humans. There has been a single report in alpine musk deer (*Moschus sifanicus*) in China (People's Rep. of). Inoculation of a positive RHDV tissue suspension 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) (Gould *et al.,* 1997). On the contrary, typical lesions and death were observed by challenging laboratory rabbits with RHDV2 positive extracts from two small rodents, the Mediterranean pine vole (*Microtus duodecimcostatus*) and white-toothed shrews (*Crocidura russula*), that were found positive for RHDV2 by RT-PCR. RHDV2 infection has also been reported in badgers (*Meles meles*) in Portugal.

The emergence of RHDV2 has drastically modified the global epidemiological situation for RHD, mainly because it is a serotype distinct from RHDV and can cause disease in young rabbits and in hares. Nowadays RHDV2 is associated with almost all RHD cases detected worldwide. Since 2010, RHDV2 quickly spread to North Africa and northern Europe and has been reported in West Africa. It also became endemic in Australia where it is replacing RHDV. In addition, several RHD outbreaks caused by RHDV2 have been reported in European rabbits and wild *Lepus* and *Sylvilagus* species in North and Central America.

RHD is characterised by high morbidity but with a variable mortality rate depending on the type of virus and the age of the rabbit. For infection with RHDV/RHDVa, mortality is around 80–90% and 5–10% of rabbits show a subacute or chronic clinical course. Although rabbits of all ages can be infected, the infection is subclinical in animals younger than 6–8 weeks of age. The disease caused by RHDV2 could last slightly longer and the mortality rate is highly variable (50–80%) depending on the strain; the most recently detected strains (from 2014 to 2015) have proven progressively more virulent than those initially identified in 2010–2011 (Capucci *et al.*, 2017). Death may occur even in unweaned rabbits from 7–15 days of age onwards.

The RHD incubation period varies between 1 and 6 days. In acute cases, infected animals develop high fever (>40°C) and die suddenly within 12–36 hours of its onset. The only clinical signs may be terminal squeals followed rapidly by collapse and death. Subclinical chronic RHD is characterised by generalised jaundice, loss of weight and lethargy. Death may occur within 1–2 weeks, but some rabbits survive after seroconversion. A specific and relevant IgM response appears within 3 days, immediately followed by an IgA and IgG response 2–3 days later. Viral RNA is detected using PCR in the blood and faeces of convalescent rabbits up to 15 weeks after the infection, as well as in

rabbits infected with RHDV but already protected by specific antibodies previously acquired (i.e. vaccinated or survivors of infection) (Gall *et al.,* 2007). Whether this is a consequence of a slow viral clearance or indicative of a real and prolonged virus replication (persistence) is yet to be established.

Following RHD serology testing, several non-pathogenic RHDV-related lagoviruses (rabbit calicivirus – RCV) have been isolated and partially characterised in Europe (Capucci *et al.,* 1996; Le Gall-Recule *et al.,* 2015; Marchandeau *et al.,* 2005), and Oceania (Strive *et al.,* 2009). Non-pathogenic lagoviruses induce a serological response that may interfere with and complicate RHD serological diagnosis (Capucci *et al.,* 1991; Cooke *et al.,* 2000; Robinson *et al.,* 2002). Recently, non-pathogenic lagoviruses have been detected in hares in Europe and Australia (Cavadini *et al.,* 2016; Droillard *et al.,* 2018; Mahar *et al.,* 2019).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of rabbit haemorrhagic disease and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
ELISA	+	–	++	+++	+	–
EM	–	–	–	++	–	–
HA	–	–	–	+	–	–
RT-PCR	+	–	++	+++	+	–
Detection of immune response						
C-ELISA	+++	+++	+++	+	+++	+++
IsoELISA	++	+++	++	+	++	++
HI	++	++	++	–	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; HA = haemagglutination test;
RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive ELISA;
isoELISA = isotype ELISA; HI = haemagglutination inhibition test.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

The liver of RHD-affected rabbits contains the highest viral titre (from $10^3 \, LD_{50}$ [50% lethal dose] to $10^{6.5} \, LD_{50}$/ml of 10% homogenate and from 10^7 to 10^{10} copies of the genome for mg of tissue) 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 is suitable while serum may serve as alternative diagnostic material.

In the case of a subacute or chronic form of RHD, the antibody response triggers virus-clearance in the liver and spleen of rabbits, so that RHD virus-like particles (VLPs) are detected instead of RHDV, mainly in the spleen but also in the liver (Capucci *et al.,* 1991). 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 and consequently it is negative in the haemagglutination (HA) test as well as with anti-RHDV monoclonal antibodies (MAbs) directed to outer conformational epitopes (Capucci *et al.,* 1995).

The initial treatment of the diagnostic samples is almost identical irrespective of the diagnostic method to be applied, except for immunostaining techniques. An organ fragment is mechanically homogenised in 5–20% (w/v) phosphate buffered saline solution (PBS), pH 7.2–7.4, and clarified by centrifugation at 5000 *g* for 10–15 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 also be directly extracted from tissues. Considering the high viral load of RHDV-positive samples and the high analytical sensitivity of PCRs methods, careful precautions must be adopted in the pre-analytical phase of sample preparation in order to avoid problems of cross contamination between samples.

1.1. Enzyme-linked immunosorbent assay

Virus detection by ELISA relies on a 'sandwich' technique and several variations of this have been described. 10% liver homogenates from RHD-affected rabbits tested positive in dilutions from 1/100 to 1/10000 with these ELISAs. Therefore, in spite of the limited sensitivity of ELISA in comparison with PCR techniques, ELISA is the best method for diagnosing acute RHD. One procedure uses the reagents, solutions, times and temperature that are used in the competitive ELISA (C-ELISA) for serology (see Section B.2.2). The microplate used should be of high adsorption capability. 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.2.2). Anti-RHDV MAbs have been produced in several laboratories and can be used instead of rabbit polyclonal sera. MAbs recognising specific epitopes expressed only by the RHDVa variant as well as by RHDV2 have also been produced (Le Gall-Recule *et al.,* 2013).

To type the RHDVs and relative variants present in the samples (RHDV, RHDVa or RHDV2) by sandwich ELISA, it is advisable to test each sample in at least four replicates, and then to use HRPO conjugates with different specificity, i.e. MAbs recognising antigenic determinants present on the virus surface and expressed alternatively by the classical strain, by the RHDVa or by RHDV2, and a pool of MAbs recognising internal epitopes that can detect smooth, degraded VLPs as well as EBHSV. Similar antigen-capture ELISAs have been described for the detection of either RHDV (Collins *et al.*, 1996) or RHDV2 (Dalton *et al.,* 2018).

1.1.1. Test procedure (example)

For steps that are not specifically indicated see the procedure of the C-ELISA for serology (Section B.2.2).

i) Coat the plate with anti-RHDV hyperimmune serum, with anti-RHDV2 hyperimmune serum and the negative RHDV serum. The latter serves as control for nonspecific reactions (false-positive samples). For each sample, eight wells must be sensitised with the positive sera and four wells with the negative one.

ii) Dilute the liver extract to 1/5 and 1/30 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 specific HRPO conjugates.

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% H_2O_2. The reaction is stopped after 5 minutes by the addition of 50 µl of 1 M H_2SO_4.

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 these agents, 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 (Capucci *et al.*, 1991; 1995), or specific EBHSV MAbs, instead of rabbit serum, is highly recommended (Capucci *et al.*, 1991).

1.2. Nucleic acid recognition methods

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 as described by several authors (Gould *et al.*, 1997; Guittre *et al.*, 1995; Yang *et al.,* 2008). This method is carried out on organ specimens (optimally liver or spleen), urine, faeces and sera using different oligonucleotide primers derived from the capsid region of the RHDV genome. The WOAH Reference Laboratory for RHD uses a single-step RT-PCR, with the following primers specific for the VP60 gene: forward: 5'-CCT-GTT-ACC-ATC-ACC-ATG-CC-3'; reverse: 5'-CAA-GTT-CCA-RTG-SCT-GTT-GCA-3'; the primers are able to amplify all RHDV variants including RHDV2. For the amplification of RHDV2 only, specific primers should be used i.e. "14U1" (5'-GAA-TGT-GCT-TGA-GTT-YTG-GTA-3') and "RVP60-L1" (5'-CAA-GTC-CCA-GTC-CRA-TRA-A-3'), which amplify a 794 bp sequence located in the C-terminal of the gene encoding VP60 of RHDV2 (Le Gall-Recule *et al.,* 2013) or "Fra109-F" (5'-ACT-ACT-AGC-GTG-GTC-ACC-ACC-3') and "Fra567-R" (5'-TTG-TTA-TAA-ACG-CTC-AGG-ACC-AAC-3'), which amplify a 481 bp sequence located in the first part of the VP60 gene (Velarde *et al.,* 2017). Viral RNA can be directly amplified using a one-step standard RT-PCR or retrotranscribed firstly into cDNA and then amplified by PCR. To visualise the PCR product, the amplified DNA is subjected to electrophoresis on agarose gel. If needed, specificity of the PCR product can be determined by sequencing.

A similar RT-PCR method has been used to identify the non-pathogenic RCV (Capucci *et al.*, 1998) or HaCV (Cavadini *et al.*, 2016; Droillard *et al.,* 2018; Mahar *et al.,* 2019) using universal primers for lagoviruses (Strive *et al.,* 2009). RT-PCR represents an extremely sensitive method for the detection of RHDV and is at least 10^4-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, RT-PCRs for the detection of EBHSV, using different primers pairs, have been applied to the detection and characterisation of EBHSV strains (Le Gall-Recule *et al.*, 2001; Velarde *et al.*, 2017).

An internally controlled multiplex real-time RT-PCR using fluorogenic probes and external standards for absolute RNA quantification has been developed as a further diagnostic tool for the detection of RHDV (Gall *et al.,* 2007). The oligonucleotides used in this method are: [VP60-7_forward: 5'-ACY-TCA-CTG-AAC TYA-TTG-ACG-3', vp60-8_reverse: 5' TCA-GAC-ATA-AGA-AAA-GCC-ATT-GG-3'] and probe [VP60-9_fam 5'-FAM-CCA-ARA-GCA-CRC-TCG-TGT-TCA-ACC-T-TAMRA-3'].

Real-time RT-PCRs specific for the detection of RHDV2 have been also developed. In the one described by Duarte *et al.*, 2015, the oligonucleotides used are: [RHDV2-F: 5'-TGG-AAC-TTG-GCT-TGA-GTG-TTG-A-3', RHDV2-R: 5'-ACA-AGC-GTG-CTT-GTG-GAC-GG-3'] and the probe [RHDV2: 5'-FAM-TGT-CAG-AAC-TTG-TTG-ACA-TCC-GCC-C-TAMRA-3'. Other real-time RT-PCR protocols have been used and are described in literature.

1.3. Electron microscopy (EM)

EM should preferably be performed after ultracentrifugation (at least 100,000 *g* for 30 minutes) of the sample (an organ suspension prepared as described in Section B.1) to concentrate the viral particles. The pellet obtained is resuspended in PBS or distilled water, put on to a grid for a few minutes, and then negatively stained with 2% sodium phosphotungstate (NaPT), pH 6.8, for 1.5 minutes. 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 (Capucci *et al.*, 1991).

Immuno-EM (IEM) which employs the same serological principles as ELISA in an EM technique has been reported for its usefulness in diagnosis and characterisation of various viruses, including RHDV. IEM, which works well with antisera or MAbs, can be more specific than traditional negative staining EM through the combination of morphological identification and antigen specificity (Lavazza *et al.,* 2015).

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.

1.4. Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (Liu *et al.*, 1984). As RHDV2 showed an HA activity similar to RHDV/RHDVa (Le Gall-Recule *et al.,* 2013) this method could be used also for RHDV2 diagnosis. HA test 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 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 VLPs.

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 VLPs (Capucci *et al.*, 1991).

Due to the practical difficulty of obtaining and keeping human red cells and the risk from working with these cells, and because of the difficulty of obtaining consistent results, this test should be replaced by other virological methods such as antigen-detection ELISA or PCR.

1.5. Immunostaining

Tissue fixed in 10% buffered formalin and embedded in paraffin can be immunostained using standard methods by employing specific MAbs (Neimanis *et al.,* 2018).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic hepatocytes in the liver, mainly in the periportal 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, in renal mesangial cells and in the bone marrow, which may exhibit a marked decrease in the proportion of myeloid to erythroid cells and an increase in the proportion of immature myeloid cells (Stoerckle-Berger *et al.*, 1992) (decreased myeloid-to -erythroid ratio) (Neimanis *et al.,* 2018).

Tissue cryosections fixed in methanol or acetone 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.

1.6. 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 can be used for determining the final diagnosis, while modern genome detection methods (real-time RT-PCR) are particularly useful for confirmation.

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 RHDV-VP60 structural protein 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.*, 1995).

Western blot analysis can also be used to identify EBHSV. 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.

1.7. Rabbit inoculation

As no efficient *in-vitro* replication system has been established for RHDV and EBHSV, cell culture isolation cannot be included among the diagnostic methods. Rabbit inoculation therefore remains the only way of isolating, propagating and titrating the infectivity of the RHDV. However, **this method should be avoided on welfare grounds for routine diagnosis**. When a case can be made for this procedure, 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). 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 (p.i.), followed by death in more than 80% of inoculated animals, depending on the type and virulence of the strain. A few individuals may survive until 6–8 days after infection. Animals that survive the disease show only a transient hyperthermia, depression and anorexia, but present a striking seroconversion that can be easily detected 3–4 days post-infection.

2. Serological tests

Infection with RHDV can be indirectly diagnosed in animals that have survived infection 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. Considering the antigenic difference existing between RHDV/RHDVa and RHDV2, distinct specific antibody responses following infection or homologous vaccination are induced. As a consequence, serological diagnosis should be based on methods using RHDV- and RHDV2-specific immunological reagents. Therefore, especially when no or limited anamnestic or epidemiological information is available, tests for both RHDV and RHDV2 should be performed, and the results compared.

Three basic techniques are applied for the serological diagnosis of RHDV: haemagglutination inhibition (HI) (Liu *et al.*, 1984), 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 (Cooke *et al.,* 2000).

Some other additional tests (Cooke *et al.*, 2000) could be used for particular investigations, and when a higher level of sensitivity is needed or to detect antibodies induced by cross-reacting non-pathogenic RCVs (see Section A. Introduction).

They are:

- *I-ELISA:* the antigen, an RHDV-positive liver homogenate, is linked to the solid phase by an MAb, the epitope of which is located on the outer shell of RHDV. The sera are then serially diluted starting from 1/40, and IgG bound to the antigen is detected using a reagent, preferably an MAb anti-rabbit IgG labelled HRPO. This ELISA has a 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 RHDV antibodies and has high sensitivity and low specificity. For these reasons it can also be used for EBHSV serology. Together with I-ELISA, this test could be considered lagovirus specific, i.e. able to detect antibodies towards common lagovirus epitopes present in the NH2 half of VP60s.

- *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 using HA or ELISA methods. In addition to the use of RT-PCR, a high level of RHDV-specific IgM and a low level, if any, of IgG are the unambiguous markers of positivity for RHD.

2.1. 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 a 25% (w/v) kaolin suspension (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.

2.1.1. 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.

As with the HA test (section B.1.4.) the difficulty of obtaining and working with human Group O blood cells has led to this test being superseded by the serological or antibody-detection ELISA.

2.2. Competitive enzyme-linked immunosorbent assay

Antigen: Due to the recent emergence of RHDV2, RHD serology should be based on the use of two antigens – classical RHDV and RHDV2.

The antigen can be prepared as described previously for HI (Section B.2.1), 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 or anti RHDV2 titre can be obtained in different ways. Two possible and currently used methods are as follows:

i)   Rabbits older than 10 weeks of age are vaccinated with a vaccine homologous to the polyclonal serum that you want to produce (RHDV or RHDV2). To obtain sera containing a high level of anti-RHDV IgG, they are orally challenged 8 days later with 2 ml of a RHDV or RHDV2 positive 10% liver homogenate diluted 1/20 in PBS. Rabbits must be bled 35–45 days post-challenge to obtain the convalescent sera (titre in C-ELISA of around 1/10240). Alternatively, convalescent rabbits can be re-infected after 3–4 months and bled 10–15 days later to obtain RHDV hyperimmune sera. In the case of RHDV2, to obtain high titre immune sera it is advisable to use strains identified from 2015 onwards (i.e. high virulent isolates).

ii) The antigen (RHDV or RHDV2) is purified from the livers of naturally or 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.*, 1991; 1995; Ohlinger *et al.*, 1990). Then the purified RHDV antigen can be used to immunise sheep, goats or chickens according to classical protocols using oil adjuvants. The same procedure can also be used to inoculate rabbits, but of course the purified virus must be 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 titrated 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.

2.2.1. Test procedure (example)

NB: This procedure is also valid for RHDV2 using the homologous reagents.

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 calculated 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% H_2O_2. Stop the reaction after 5 minutes by addition of 50 µl/well 1 M H_2SO_4.

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.

The above criteria used to transform raw ELISA data into final serological results are the same for the RHDV and RHDV2 C-ELISAs. However, for a practical interpretation of the obtained results, some considerations must be borne in mind. The main one is that RHDV and RHDV2, although representing two distinct serotypes, share secondary antigenic determinants. These determinants induce a minor subset of cross-reactive antibodies that, although of limited importance in RHD protection, 'interfere' in ELISA reactions. This means that rabbits vaccinated (or infected) with RHDV will have medium or high titres in the homologous C-ELISA but will be positive to some extent in the heterologous C-ELISA (RHDV2 C-ELISA). The opposite is also true, when rabbits are vaccinated or infected with RHDV2 and tested with RHDV C-ELISA. However, to ascertain which vaccine was used (or which virus infected the rabbits) it may be possible to use the value obtained by the 'ratio' RHDV2 C-ELISA titre divided by the RHDV C-ELISA titre (RT2 value) (Velarde *et al.,* 2017). This value usually ranges from 4 to 64 in rabbits vaccinated or infected with RHDV2 and from 0.25 to 0.0156 in rabbits vaccinated or infected with RHDV. For an RT2 value from 2 to 0.5, it is not possible to assign the origin of the detected antibodies to one or the other viruses. This could occur when both titres are low (<1/80) or when rabbits are vaccinated with a bivalent vaccine (RHDV plus RHDV2), or two associated vaccines (RHDV and RHDV2).

Due to the significant antigenic differences existing between RHDV and EBHSV, 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.

2.3. Isotype enzyme-linked immunosorbent assays (isoELISAs)

The isoELISAs enable the detection and titration of isotypes IgA, IgM and IgG. Isotype titres are critical for the interpretation of field serology in four main areas: cross-reactive antibodies, natural resistance of young rabbits, maternal antibodies, and antibodies in previously infected rabbits (Cooke *et al.*, 2000). In

fact, in the case of passive antibodies, only IgG are detected; in vaccinated animals, no IgA are usually 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 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.2.2, 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 H_2SO_4 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 OD_{492} (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. This method could be applied also for serology with RHDV2, obviously using the RHDV2 specific MAbs.

C. REQUIREMENTS FOR VACCINES

1. Background

In countries where RHD is endemic, indirect control of the disease in farmed animals and pet rabbits is achieved by vaccination. Most of the commercially available vaccines are based on inactivated infectious viruses. Due to the lack of a cell culture system for efficient virus propagation, such vaccines are 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, aluminium hydroxide or other emulsions), can vary according to the protocol used by the different manufacturers.

The level of cross protection induced by vaccination with RHDV/RHDVa vaccine against RHDV2 is poor and does not prevent infection and losses due to clinical disease. Therefore, combined vaccination with both antigenic types and/or vaccines homologous to the RHDV type identified during the epidemics or the outbreak should be used. Considering the current variability of RHDV2 strains, the use of vaccines based on strains showing a high homology with those circulating in an area/region/country is highly advisable.

Most vaccine manufacturers recommend a single basic vaccination, with yearly booster. Usually, a 1-ml dose is inoculated subcutaneously in the neck region, or intramuscularly. In those units with no history of disease, with negative serology for RHD, it is advisable to vaccinate only the breeding stock. Considering the high restocking rate in industrial rabbit farms, the usual vaccination programme is to administer the vaccine to all breeders, independently of their age, every 6 months. This should ensure that all animals get at least one vaccination per year. Booster vaccination 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, up to the age of 6–8 weeks, to the disease caused by RHDV/RHDVa, but not by RHDV2, vaccinating these rabbits is not necessary if good biosecurity measures are applied on farm and there are no outbreaks of the disease in the area. Following an outbreak of RHD, and especially in the case of RHDV2, which could induce disease even in young animals, 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 30–40 days, because the incidence of re-infection is very high. Only after several (>3) production cycles it is 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 applied. Indeed, better results in limiting the spread 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. In both situations (vaccination followed by post-exposure treatment and passive protection with hyperimmune sera), it is necessary to use vaccine and sera homologous to the causative RHDV strain. This is particularly true in the case of RHDV2 given the poor cross-protection induced by classical vaccines based on RHDV/RHDVa.

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

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

At present, RHDV replication can only be produced by infection of 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. More variability could be encountered when obtaining livers for RHDV2 vaccine because of the different level of mortality registered in experimental infections depending on the virulence of the strain used.

2.1.2. 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 re-suspended 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 re-suspended in PBS (1/100 of the starting volume).

This viral suspension is then characterised by any of the following methods: negative-stain EM examination, determination of reactivity in ELISA with different specific MAbs, and HA activity at room temperature (HA titre against RBCs of human Group O higher than 1/1280).

The absence of viable bacteria, or fungi should be determined by using common laboratory bacteriological methods. PCR methods may be used for the detection of mycoplasma and of rabbit specific extraneous viruses (e.g. *Myxoma* virus).

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 a variable death rate among animals according to the type of strains, i.e. 70–80% of the rabbits in the case of RHDV/RHDVa and up to 80% in the case of RHDV2 depending on the virulence of the strain, within 24–96 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 intercurrent diseases.

Seed virus is titrated before use and should contain at least 10^5 LD$_{50}$. It should be stored frozen (–70°C), better with the addition of 1:1 volume of glycerol or freeze-dried.

2.1.3. Validation as a vaccine strain

Due to the lack of effective cross protection when using heterologous vaccines (i.e. RHDV/RHDVa vs RHDV2), for vaccine preparation it is recommended to use those strains that are highly homologous with the dominant RHDV serotype causing outbreaks in an area.

It is also important to have current and homologous vaccine controls because of induced immunity with respect to the expressed antigenic pattern of the field strains.

2.1.4. Procedure for provisional acceptance of new master seed virus

Current epidemiological data clearly show that RHDV2 has almost replaced classical RHDVs, and thus there is an increasing demand for RHDV2 vaccines. As RHDV2 is a 'new' emerging virus and not just a genetic variant of RHDV/RHDVa, it has undergone a significant evolution since 2010 resulting in changes in virulence and in antigenic profile (Capucci *et al.,* 2017). As a consequence, it is recommended to choose as master seed for vaccine production one of those strains isolated from 2015 onwards, and to base the selection on their antigenic profile.

Nevertheless, it should be remembered that while wild and domestic rabbit populations are rapidly gaining a high herd immunity against RHDV2, they are simultaneously losing any protection against classical RHDV/RHDVa strains that have not completely disappeared. This fact should be considered in the design of RHD surveillance systems so that countries can be ready to react promptly to epizootics by updating vaccine composition.

2.2. Method of manufacture

2.2.1. Procedure

The vaccine manufacturing procedure for both antigenic types (RHDV and RHDV2) follows a similar protocol. Following inoculation of susceptible rabbits, the liver and spleen of those rabbits that die between 24 and 96 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 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.

2.2.2. Requirements for ingredients

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 and biosecure health conditions (see Laboratory animal facilities in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary 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$_{50}$.

2.2.3. In-process controls

i)   Antigen content

The RHDV 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. The identity of RHD could be also confirmed by negative-staining EM or real-time RT-PCR analyses.

ii)   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.

iii)   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.

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 the C-ELISA method specific for the homologous virus) and those injected with livers obtained after the first passage should be serologically negative.

2.2.4. Final product batch tests

Batch release safety tests are not required, except in the case of autogenous vaccines. Sterility, 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.

i)   Sterility/purity

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.

iii)   Safety

Where safety tests are required, the following procedure should be carried out:

a)   The safety of the administration of one dose;

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

c)   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 at least 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.

iii) 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. Five 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 five 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 not 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) following challenge with RHDV/RHDVa, less than 60% of control rabbits died with typical signs of RHD; or c) following challenge with RHDV2, less than 20% of control rabbits die and less than 60% of them show high antibody titres (>1/1280 using the homologous C-ELISA). The vaccine complies with the test if: a) not less than 80% 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.

2.3. Requirements for regulatory approval

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.

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.2 *Method of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Rabbit is the sole species susceptible to RHDV (with the exception of some hare species susceptible to RHDV2) 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 rabbits born, and the number of live rabbits born; percentage of mortality at the time of weaning; average weight of young rabbits 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 health or abnormal local or systemic reactions for the whole test duration.

ii) Reversion-to-virulence for attenuated/live vaccines

Not applicable.

iii) Precautions

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.

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.

2.3.3. Efficacy requirements

The efficacy should be tested in the laboratory with both challenge and serology tests. Twenty rabbits (10 vaccinated and 10 unvaccinated), at least 4 months of age, are challenged with virulent virus: at least 90% of the vaccinated animals must be protected, giving positive serological titres and a proportion of the control unvaccinated animals similar to that naturally recorded according to the type of strain (i.e. 70–90% for RHDV and 50–80% for RHDV2) must have died within the observation period.

The in-field efficacy of the vaccine may be determined by evaluating the seroconversion in blood samples taken from both fattening and breeder rabbits at different check-points from vaccination. Titres are measured by C-ELISA and anti-isotype IgM, IgA and IgG ELISAs, by using specific and homologous methods according to the type of virus (RHDV/RHDVa or RHDV2).

Before the first vaccination, the C-ELISAs should confirm, in all rabbits, the absence of anti-RHDV antibodies. 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 adjuvant 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 parenterally 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 titres obtained by C-ELISAs and the state of protection from the disease induced by the different strains (see Section B.2.2.) taking into account the RT2 value found, i.e. rabbits with antibodies titres specifically induced by one strain (RHDV/RHDVa/RHDV2) did not show any sign of disease when challenged with the same virulent strain. 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 for 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. In young rabbits (<35–40 days old), a low level of antibody (1/80–1/320) could also be induced by an active infection with RHDV/RHDVa not leading to disease, as commonly occurs in animals of this age.

2.3.4 Duration of immunity

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.

2.3.5 Stability

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

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

3. Vaccines based on biotechnology

Biotechnology-based approaches for vaccine production have been, and are being, developed. The aim will be to circumvent welfare concerns with conventional vaccine production, to avoid biosecurity risks, and to provide a broad spectrum protection against multiple strains of the virus.

Several studies have been carried out on the expression of RHDV/RHDVa/RHDV2 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 and or RHDV2 capsid protein were protected against lethal RHDV and MV challenges.

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, none of these vaccines has been registered and therefore, they are not commercially available.

A vaccine 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, has been developed in France and then marketed in some European countries.

Recently, two new biotechnology vaccines, one bivalent and the other trivalent have been developed and registered and are commercially available in several countries for administration by the parenteral route. In the first, the RHDV or RHDV2 VP60 gene has been inserted into the genome of a vaccine strain of myxomatosis virus, which, when inoculated into the rabbit, replicates, also producing RHDV VP60. The second vaccine is based on the same principle, contains two different myxoma virus strains each producing the VP60 of RHDV and RHDV2, respectively (Reemers *et al.,* 2020). These vaccines need approximately 3 weeks to induce detectable antibodies.

In addition, another bivalent vaccine against RHDV and RHDV2, in which the viral capsid is expressed in the form of virus-like particles from a baculovirus growing on the pupae of *Lepidoptera* (Dalton *et al.,* 2021), has been approved by the European Medicines Agency and should soon be available on the market. These recombinant-type vaccines do not need the inactivation step, and only the one based on the baculovirus system uses aluminium hydroxide as

adjuvant. Similarly to inactivated organ vaccines, this vaccine induces a quick immune response and antibodies are detectable at 7–10 days post-vaccination.

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NB: There is a WOAH Reference Laboratory for rabbit haemorrhagic disease (please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for rabbit haemorrhagic disease

NB: First adopted in 1991 as Viral haemorrhagic disease of rabbits.
Most recent updates adopted in 2023.