RABBIT HAEMORRHAGIC DISEASE

SUMMARY

Description of the disease: *Rabbit haemorrhagic disease* (*RHD*) *is a highly contagious and acute fatal hepatitis of the European rabbit* (Oryctolagus cuniculus), *caused by a calicivirus* (*genus* Lagovirus)*. Up to 2010, all RHD viruses* (*RHDV*) *isolated belonged to one of the six identified genotypes* (*GI–GVI*), *among which the GVI is an antigenic subtype* (*RHDVa*)*. In 2010, an additional RHDV was identified, phylogenetically and antigenically distinct from RHDV and provisionally called RHDV2 or RHDVb. A similar disease,* termed *European brown hare syndrome* (*EBHS*)*, has been described in the hare* (Lepus europaeus)*.*

RHD is characterised by high morbidity and a mortality of 70–90% for RHDV/RHDVa and 5–70% for RHDV2. Infection mainly occurs by the oral route. In wild rabbits in particular, insects are considered an important route of infection or transmission, and are often the source of long-distance spread. 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, apathy 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 15 to 20 days of age onwards.

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 RHDV, RHDVa and RHDV2. 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 anti-RHDV antibodies confers a full protection from the disease. The best RHD serological methods are based on the competitive ELISA using specific anti-RHDV 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, due to the deformation of the viral capsid when adsorbed to the plate, the exposition of the internal common epitopes among RHDVs and RCVs* (*rabbit calicivirus*)*, decreases the test's specificity.*

Requirements for vaccines: *Indirect control of the disease is easily achieved by vaccination. Although the capsid RHDVs has been expressed as recombinant VPLs, as of 2015 the vaccines used are inactivated vaccine prepared from livers of experimentally infected rabbits, and thus inactivated and adjuvanted. Vaccinated animals quickly produce solid protective immunity against*

RHDV infection (within 7–10 days) and experimental data indicate that protection lasts for a long period (over 1 year). As RHDV2 is a distant antigenic subtype or even a second RHDV serotype, combined vaccination with both antigenic types or the use of a vaccine homologous to the RHDV 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 wild and domestic European rabbits (*Oryctolagus cuniculus*). 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, very similar to RHD. Despite their high genetic relationship (VP60 nucleotide similarity of 70%), RHDV and EBHSV are two distinct viral species (Lavazza *et al.*, 1996; Wirblich *et al.*, 1994).

Genetic and antigenic comparison and epidemiological data indicate the existence of three main RHDV groups:

i) The "classical RHDV", including genogroups G1–G5, first reported in China (People's Rep. of) in 1984 (Liu *et al.*, 1984). To date, this RHDV has been reported in over 40 countries in Asia, Africa, Americas, Europe and Oceania and is endemic in most parts of the world where European rabbits live naturally or are domesticated.

ii) The subtype RHDVa/G6 identified in Europe on 1996 (Capucci *et al.*, 1998; Schirrmeier *et al.*, 1999) and currently reported in other continents (Oceania, Asia and Americas).

iii) The "new" RHDV (provisionally called RHDV2 or RHDVb) emerged in France in 2010 in wild and farmed vaccinated rabbits (Dalton *et al.*, 2012; Le Gall-Reculé *et al.*, 2001, 2013), and rapidly spread in Europe and the Mediterranean basin (Malta and Tunisia), and also in Australia in 2015. According to Le Gall-Reculé *et al.* (2013), RHDV2 did not derive from classical RHDV, but rather it originated as a second emergence of unknown origin. Antigenic and disease protection data led to it being classified RHDV2 as a second RHDV serotype.

RHD is characterised by high morbidity but a variable proportion of rabbits die according to the type of virus. The incubation period of RHDV/RHDVa is 1–3 days, and death usually occurs 12–36 hours after the onset of fever (80–90% lethality). Around 5–10% of rabbits show a subacute-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 has typical features: the mortality rate is lower but highly variable (5–70%) with an average mortality of 20% in experimentally infected rabbits; death occurs in lactating rabbits from 15 days of age onwards and the course of the diseases is usually longer (3–5 days), with higher proportion of rabbits showing subacute-chronic disease. In addition, RHDV2 is able to cause an RHD-like disease in two hare species: Cape hare (*Lepus capensis* var. *mediterraneus*) (Puggioni *et al.*, 2013) and Italian hare (*Lepus corsicanus*) (Camarda *et al.*, 2014).

Subclinical-chronic RHD is characterised by severe and generalised jaundice, loss of weight and lethargy. Death may occur after 1 or 2 weeks, but some rabbits survive after seroconversion. Specific and relevant IgM response appears within 3 days, immediately followed by an IgA and IgG response 2–3 days later (Barbieri *et al.*, 1997). The viral RNA is detected using polymerase chain reaction (PCR) up to 15 weeks after the infection (Gall *et al.*, 2007) 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). 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 non-pathogenic RHDV-related lagoviruses (rabbit calicivirus – RCV) have been isolated and partially characterised in Europe and Oceania (Capucci *et al.*, 1996; 1997; Forrester *et al.*, 2002; Marchandau *et al.*, 2005; Strive *et al.*, 2009; White *et al.*, 2004). These "enteric viruses" induce a serological response that may interfere with and complicate RHD serological diagnosis (Capucci *et al.*, 1991; Cooke *et al.*, 2000; Marchandeau *et al.*, 2005; Nagesha *et al.*, 2000; Robinson *et al.*, 2002).

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
Agent identification[1]						
Antigen detection	+	–	++	+++	+	–
Real-time RT-PCR	+	–	++	+++	+	–
Detection of immune response						
ELISA	+++	+++	+++	–	+++	+++
HA	++	++	++	–	++	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = Virus neutralisation; HA = haemagglutination test.

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 is useful while serum may serve as alternative diagnostic material,

In the case of animals that die from a subacute or chronic form of RHD the antibody response triggers virus-clearance in the liver and spleen of rabbits, so that an RHD virus-like particle (VLP) is detected instead of RHDV, mainly in the spleen but also in the liver (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 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, 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. 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.

1.1. 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.2.2). 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–

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1   A combination of agent identification methods applied on the same clinical sample is recommended.

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 (Capucci, pers. data; Le Gall-Reculé *et al.,* 2013).

To type the RHDVs 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. 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.*, 1996).

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 (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 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 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 (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/RHDVa 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). 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 OIE 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-Reculé *et al.,* 2013). cDNA obtained from the RT reaction is usually PCR amplified as described (Guittre *et al.*, 1995) or using one step standard RT-PCR methodology. 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. A similar RT-PCR method has been used to identify the non-pathogenic RCV (Capucci *et al.*, 1998). 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 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® 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 oligonucletides 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']. A real-time TaqMan RT-PCR specific for the detection of RHDV2 has been also developed (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 probe [RHDV2: 5'-FAM-TGT-CAG-AAC-TTG-TTG-ACA-TCC-GCC-C-TAMRA-3'.

1.3. 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 floated 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 ×19–28,500 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) 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).

For diagnostic purposes and especially when other methods give doubtful results, the best EM method is an immuno-EM technique (IEM) (Lavazza *et al.,* 2015). 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.

1.4. Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (Lui *et al.*, 1984). As RHDV2 showed an HA activity similar to RHDV/RHDVa (Le Gall-Reculé *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).

Because of 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 has been replaced by the virus-detection ELISA.

1.5. 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% H_2O_2 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).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic cells 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, and in renal mesangial cells (Stoerckle-Berger *et al.*, 1992).

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 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.*, 1996).

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 (polyvinylidene 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 A 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-indolylphosphate 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.

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. When using RHDV/RHDVa as inoculum a rise in body temperature is registered between 18 and 24 hours post-infection (p.i.), followed by death in 70–90% of cases. A few individuals may survive until 6–8 days after infection. When testing the *in-vivo* pathogenicity of RHDV2, mortalities are much lower (on average 20%), occur later and over a longer period than with both classical RHDV and RHDVa, i.e. 3–9 days p.i. and lasting 5 days instead of 2–6 days post-inoculation and lasting 3–4 days as generally observed with classical RHD. Animals that survive 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. 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) (Lui *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 (Capucci, unpublished data; 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, a 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 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.

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 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.

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 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. In the case of RHDV, due to its high mortality rate, it is necessary to infect at least 10–15 seronegative rabbits older than 8 weeks of age, 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 (titre in C-ELISA of around 1/10240). Alternatively, convalescent rabbits can be re-infected 3–4 months post-infection and bled 10–15 days later to obtain RHDV hyperimmune sera. In the case of RHDV2, the main difficulties are related to evidence that the sera of infected convalescent rabbits often have a titre 20–40 times lower than that induced by RHDV, presumably as a consequence of the low mortality induced. Therefore, as in the previous case, at least 10–15 seronegative rabbits have to be used for the experimental infection.

ii) The antigen (RHDV or RHDV2) 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.*, 1991; 1995; Ohlinger *et al.*, 1990). Then the purified RHDV antigen 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 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 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% 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. This value was shown to be valid also for C-ELISA with RHDV2.

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.*, 1991; 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.

2.3. Isotype enzyme-linked immunosorbent assays (isoELISAs)

These 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 resistence of young rabbits, maternal antibodies, 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 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.2.2, 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

1.1. 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.

The level of cross protection induced by vaccination with RHDV/RHDVa vaccines against RHDV2 is poor and does not prevent infection and losses due to clinical disease. Therefore combined vaccination

with both antigenic types or the use of a vaccine homologous to the RHDV strain identified during the epidemics or the outbreak, is highly advisable.

Most vaccine manufacturers recommend a single basic vaccination, with yearly booster. Normally, a 1-ml dose is inoculated subcutaneously in the neck region, or intramuscularly. The first injection should be given at 2–3 months. 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 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. 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 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 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

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 difficulties could be encountered when obtaining livers for RHDV2 vaccine because of the lower mortality registered in experimental infections.

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 three 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, fungi or mycoplasma should be determined by using common laboratory bacteriological methods. PCR methods may be used for the detection of 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 lower in the case of RHDV2 (20% on average), 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.

2.2. 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 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 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. Negative-staining EM confirms the identity of RHD.

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

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.

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

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:

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. 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 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 70% of control rabbits died with typical signs of RHD; or c) following challenge with RHDV2, less than 10% of control rabbits die and less than 70% of them show high antibodies titres (>1/1280 using the homologous C-ELISA). 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.

2.3. 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.

2.3.1. Safety requirements

i)   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 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.

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.

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

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

iii) 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.

2.3.2. 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: 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 5–70% 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 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 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 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 obtained by each C-ELISA and the state of protection from the disease induced by the homologous strains, i.e. rabbits with titres higher than 1/10 for antibodies 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 (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 with RHDV/RHDVa 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.

2.3.3. 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

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. This type of recombinant vaccine, i.e. a modified *Myxoma* virus expressing the main RHDV protein, has been developed and registered, and is commercially available in several countries for administration by the parenteral route.

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.

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NB: There is an OIE Reference Laboratory for Rabbit haemorrhagic disease
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE 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 2016.