Viral Rabbit Hemorrhagic Disease

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Abstract: Rabbit haemorrhagic disease virus (RHDV) is a calicivirus of the genus *Lagovirus* that causes rabbit haemorrhagic disease (RHD) in adult European rabbits (*Oryctolaguscuniculus*). First described in China in 1984, the virus rapidly spread worldwide and is nowadays considered as endemic in several countries. Factors that may have precipitated RHD emergence remain unclear, but non-pathogenic strains seem to pre-date the appearance of the pathogenic strains suggesting a key role for the comprehension of the virus origins. All pathogenic strains are classified within one single serotype, but two subtypes are recognised, RHDV and RHDVa. RHD causes high mortality in both domestic and wild adult animals, The disease is characterised by acute necrotising hepatitis, but haemorrhages may also be found in other organs, in particular the lungs, heart, and kidneys due to disseminated intravascular coagulation. Resistance to the disease might be explained in part by genetically determined absence or weak expression of attachment factors, but humoral immunity is also important. Disease control in rabbits relies mainly on vaccination and biosecurity measures.
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Introduction

Rabbit haemorrhagic disease (RHD) is an acute and lethal form of viral hepatitis in rabbits (Oryctolaguscuniculus) with a mortality rate in adults ranging from 70 per cent to 100 per cent. RHD was first reported in China in 1984, in Europe in 1986, where it caused severe losses to rabbit, and in Australia in 1996 **(Abrantes et al., 2012)**. RHD is caused by the lagovirus RHD virus (RHDV) in the family Caliciviridae. In the course of its evolution, RHDV split into six genotypes **(Kerr et al., 2009),** all highly pathogenic and virulent. Genotype 6 is the antigenic subtype (RHDVa) that became prevalent in certain countries, including the USA **(McIntosh and et al., 2007).** In addition, other enteric non-pathogenic rabbit caliciviruses (RCVs) related to RHDV have been identified in Europe and Australia (**Capucci et al., 1996, Strive et al., 2009, Le Gall-Reculé et al., 2011a)**. The main natural transmission mechanism of RHDV is direct contact between infected rabbits through respiratory and oral routes and skin lesions **(Xu et al., 1988)**. Subclinically ill or infected rabbits are the principal sources of infection **(Xu and Chen, 1989)**. Moreover, rabbits with persistent infection **(Lo¨ liger and Eskens, 1991),** carriers (**Cancellotti and Renzi, 1991; Cooke, 2002)**, as well as young rabbits under 20 days old who manage to survive the disease **(Rosell et al., 1989)**, can also act as reservoirs of the virus. However, possible alternative hosts of the virus and their role in the epidemiology of the disease have scarcely been studied. Some field and laboratory studies have shown how predatory mammals and birds play a role in the transmission of RHDV **(Chasey, 1994; Gavier-Widen and Morner, 1993; Simo´n et al., 1994**), acting as mechanical reservoirs or vectors. **Schirrmeier et al. (1990)** conducted an experiment with rodents and insects, suggesting that these did not act as reservoirs; they did, however, act as possible passive transmitters of the virus. Likewise, other studies prove the importance of the role played by insects in transmission **(Asgari et al., 1998; Gehrmann and Kretzschmar, 1991; Lenghaus et al., 1994),** as well as that of the decomposing remains of the infected rabbits themselves **(McColl et al., 2002)**, or their warrens in housing the RHDV **(Calvete et al., 2002)**.

In 2010, a new lagovirus was identified in France. This virus showed a capsid protein sequence identity of about 80 per cent with RHDV and was able to cause RHD in vaccinated and young rabbits (15–25 days old) **(Le Gall-Reculé et al., 2011a, b).** In addition, it showed a distinct antigenic profile and induced an average mortality rate of 20–30 per cent in both experimental infections and natural cases; such a low mortality rate was never observed in the many experimental rabbit infections carried out with other strains of RHDV. The remaining 70–80 per cent of the rabbits survived the infection without showing typical signs of RHD **(Le Gall-Reculé and others 2011b).** Unexpectedly, in autumn 2011, this new virus also

caused fatal cases in cape hares (Lepuscapensis varmediterraneus) **(Puggioni et al., 2013)**. All these features strongly suggested that the virus was not derived from RHDV but rather that it had recently emerged from an unknown source; for this reason, we named it RHDV type 2(RHDV2). Within a couple of years, RHDV2 spread throughout Europe **(Le Gall-Reculé et al., 2013; Dalton et al., 2014).**

An etiology

Rabbit hemorrhagic disease virus (RHDV) is a member of the genus *Lagovirus*, family *Caliciviridae*. Only a single serotype is known, but two major subtypes exist: RHDV and the antigenic variant RHDVa. RHDVa does not currently occur in Australia. Nonpathogenic strains of RHDV also occur with an endemic Australian strain, RCV-A1, identified **(Strive et al. 2008).**

Natural hosts

RHDV is highly specific to rabbits of the genus *Oryctolagus*. Hares (*Lepus* sp.) and rabbits of other genera (*Sylvilagus*sp) do not appear to be susceptible **(The Merck Veterinary Manual 2011)**. Other mammals and some rabbit predators can develop antibodies and excrete virus, but virus replication has not been observed.

Epidemiology

Morbidity and mortality estimates for RHD have a broad range as rabbits often die in their burrows, making accurate assessments difficult. In farmed rabbits the estimated morbidity rate ranges from 30–100% with mortality rates ranging from 40–100%, however the typical mortality rate is around 90% **(The Merck Veterinary Manual 2011).** Morbidity rates in wild populations range from exceedingly low to high in the 90% range, however the mortality rate is around 70–80%. RHDV incubates for a period of 1-3 days and death may occur 1-2 days post-infection. Transmission is viadirect contact with infected animals or via fomites. Species of blowfly (Calliphoridae) and bush fly (Muscidae) are known vectors of RHDV in Australia (**Asgari *et al.* 1998, McColl *et al.* 2002),** with fresh fly-spots containing2–3 times the median lethal dose (LD50). Rabbit fleas (*Sphilopsylluscuniculi* & *Xenopsyllacunicularis*) and mosquitos (*Culexannulirostris*) may also be important **(Lenghaus, 1994).** RHDV is acquired through oral, nasal or conjunctival pathways, and seroconverted carriers may be infectious for up to a month depending on the climatic conditions. All excretions are thought to contain virus.

Host specificity

Despite concern that RHDV may not be specific to rabbits **(Smith 1998),** all information up to this stage points to the fact that conclusions drawn from challenge tests on 28 species of vertebrates in the Australian Animal Health Laboratory were correct. The European rabbit is the only host of RHDV. Even other lagomorphs that have been experimentally exposed show no clinical signs of disease; these include: the eastern cottontail (*Sylvilagusfloridanus*), black-tailed jackrabbit (*Lepus californicus*), and volcano rabbit (*Romerolagusdiazi*). The European brown hare (*Lepuseuropaeus*) and the varying hare (*Lepustimidus*) appear not to be natural hosts for RHD but are susceptible to the closely related EBHSV that causes European brown hare syndrome. Interestingly, the varying hare only becomes infected with EBHSV where its population overlaps with the European brown hare **(Gavier-Widen and Morner 1993)**. Because EBHSV cannot maintain itself in populations of varying hares alone, the varying hare seems unlikely to be the true host. **Etherington *et al* (2006)** have recently used available sequence data and phylogeneticanalysis tools to consider the evolutionary paths of caliciviruses and their hosts and conclude that caliciviruses have occasionally switched hosts but there is no evidence that caliciviruses from any other mammalian groups have entered human populations as a result of zoonoses (disease spread from wildlife). Rather, host switching of caliciviruses, such as the case of San Miguel Sea-lion Virus (SMSV), has been associated with feeding sea-lion meat to swine **(Smith *et al* 1973)** and the close genetic similarity of some human, bovine and porcine caliciviruses suggests that switches in both directions may have occurred in association with the prehistoric domestication of livestock **(Van Blerkom 2003).**

Transmission

When RHD first spread in domestic rabbits it became clear that rabbit to rabbit contact and aerosol droplet transmission between closely-spaced cages were common means of transmission. Spread between rabbitries could usually be accounted for by movement of contaminated material on footwear, foodstuffs and cages (referred to as fomites) and by poor livestock hygiene measures among people handling stud rabbits or rabbits taken to abattoirs **(Morrisse *et al* 1991). Gehrman and Kretzschmar (1991)** nevertheless showed experimentally that stable flies of the genus *Phormia* were able to transmit the virus after feeding on the conjunctiva of rabbits. Only a few viral particles were required for infection by this route.

Experimentally, RHDV is known to be transmitted not only by oral, ocular and nasal routes but also by intra-dermal and sub-cutaneous injection as well as intra-muscular inoculation. Spread

involving biting insects such as mosquitoes *Culexannulirostris* and rabbit fleas (*Spilopsylluscuniculi* and *Xenopsyllacunicularis*) can be expected on the basis of laboratory experiments **(Lenghaus *et al* 1994).** It is also known that bush flies, *Muscavetustissima*, can transmit RHDV in the laboratory by feeding on the conjunctiva of infected rabbits **(McColl *et al* 2002).**

Nonetheless, scavenging flies such as *Calliphora* spp. are the insects most closely linked to natural RHD outbreaks. Using PCR, pools of trapped flies have widely been found positive for RHDV during disease outbreaks in both Australia and New Zealand **(Asgari *et al* 1998; Barrett *et al* 1998)**. **Asgari *et al* (1998)** demonstrated the presence of virus in the faeces and crop regurgita in blow flies fed on infected rabbit livers. There were sufficient live virus particles in a single fly spot (regurgitated crop contents or faeces) to infect both domestic and wild rabbits.

Pathogenesis

The disease caused by RHDV in adult rabbits is characterized by high morbidity and mortality rates and in domestic rabbitries in Europe losses of up to 90% were reported as the virus first spread. When RHD first spread in Australia, it was estimated that the morbidity and mortality rates of wild rabbits in the Flinders Ranges were 98% and 97% respectively **(Mutze *et al* 1998).**

The time from infection to death depends on the route of infection **(Cooke and Berman 2000)**. Orally infected rabbits die about 60 hours after infection, about 21 hours later than those inoculated subcutaneously or intramuscularly. Infected rabbits become pyretic although body temperature may fall below normal in late stages of the disease **(Robinson *et al* 2002)** and death results from widespread circulatory dysfunction associated with disseminated vascular coagulation and necrotizing hepatitis. Rabbits appear to behave normally until about 12 hours before death and may continue to eat sporadically until a few hours or sometimes minutes before death.

Just prior to death, there may be intermittent short struggles, rabbits lying on their side and paddling. This may be followed by a period in which the rabbit may right itself or continue lying in a comatose state until death. Bloody mucous discharge from the nose reported in domestic rabbits prior to death from RHD has not been reported among experimentally infected wild rabbits in Australia. Internally, the liver is pale and discoloured with a reticulate pattern, the spleen is swollen and there may be haemorrhagic lesions in the trachea, lungs and occasionally the kidneys.

Gender and body weight have some influence on survival time with female rabbits dying ahead of males and heavy rabbits dying before lighter ones. However, ambient temperatures between 13ºC and 27ºC do not appear to influence the course of the disease **(Cooke and Berman 2000)** even though high environmental temperatures can have a powerful effect in reducing mortality rates for myxoma virus infection **(Marshall 1959).**

Large quantities of virus are found in the rabbit's liver and other organs and virus particles are present in discharges from the nose. In adult rabbits, virus may also be excreted in urine and faeces commencing about 36 hours after sub-cutaneous infection. In young rabbits this is further delayed to about 48 hours **(Shien *et al* 2000).**

Rabbits with acute RHD may shed virus for little more than 12 - 24 hours before dying whereas, from an epidemiological perspective, rabbits that recover from the disease may be more likely to spread virus in contaminated excreta.

At a more detailed cellular and biochemical level, programmed cell death or apoptosis of infected hepatocytes has been described **(Alonso *et al* 1998, Jung *et al* 2000)** and 16the release of hepatocyte enzymes including aspartate aminotransferase and alanineaminotransferase into the blood has been used to monitor severity of infection **(Ferreira *et al* 2006)**. **San Miguel *et al* (2006)** have shown that the administration of N-acetyl-cysteine reduces liver cell-death, probably by inhibiting the pathway of apoptosis.

Rabbits that recover from RHD appear normal severe liver damage including loss of glycogen reserves **(Ferreira *et al* 2006)** may cause prolonged ill health or death. There have been reports of 'earless rabbits', technically called chondropathy of the pinna, in association with outbreaks of RHD in colder regions of New Zealand **(Clark *et al* 1999).**

Diagnostic methods

A wide range of techniques have been used for investigating RHDV, studying its structure and following its epidemiology. These include electron microscopy, x-ray crystallography, in-situ hybridization, polymerase chain-reaction (PCR) and nested.

PCR, nucleotide sequencing, and Western blotting. Detection of antibodies in rabbit sera initially involved haemagglutination techniques but these were abandoned when a wide array of enzyme-linked immuno-sorbent assays (ELISA) was subsequently developed **(Capucci *et al* 1991).**

For epidemiological studies in the field, ELISA techniques are particularly useful for detecting virus antigens and antibodies to them. Commonly, *virus capture* ELISA (vcELISA) is used to detect viral particles, and a *competition* ELISA (cELISA) is used

to detect antibodies in rabbits that have recovered from infection with RHDV.

Antibodies can be further analysed using *isotype* ELISAs to distinguish between different types of antibodies including IgA, IgG and IgM. The ratios of these isotypes give some insight into the antibody status of rabbits. For example, young rabbit kittens with exclusively IgG antibodies are almost certainly carrying antibodies of maternal origin. Likewise, young rabbits with high IgM titres are likely to have recently recovered from RHD **(Capucci *et al* 1991, Cooke *et al* 2000).**

Vaccines against RHD

Several RHD vaccines have been produced. Most are made from inactivated virus preparations derived from the livers of infected rabbits. Vaccines such as Cylap.

HVD® use a liquid oil adjuvant to promote antibody response. Recombinant RHDV capsid protein expressed in baculovirus and emulsified in Freund's complete adjuvant also gives protection against the development of RHD within five days of inoculation **(Laurent *et al* 1994). Boga *et al* (1997)** used the RHDV major capsid protein produced in the yeast *Saccharomyces* to induce protection without an adjuvant.

There have also been attempts to use recombinant VP60 capsid expressed in potatoes under the control of cauliflower mosaic virus 35S promoter as a vaccine against RHD **(Castañón *et al* 1999)**. The product could be used to immunize rabbits if inoculated intramuscularly with a suitable adjuvant but the concentration of recombinant capsid protein was not high enough to immunize rabbits fed on the transgenic potatoes alone.

Nevertheless, **Gil *et al* (2006)** have suggested that oral immunization might be achieved if different types of plants (e.g. *Arabidosis*) and other promoters were used to increase the concentration of VLPs. Such a claim should nevertheless be taken cautiously given that fact that rabbits exposed to low doses of live RHDV frequently avoid infection without developing antibodies (see *Resistance to RHDV infection*).

While vaccines are largely used to protect domestic rabbits from RHD, a matter of greater concern for Australia and New Zealand has been the development in Spain of a genetically modified myxoma virus that expresses antigens from the coat protein of RHDV. This was developed as a means of simultaneously immunizing wild rabbits against both diseases **(Torres *et al* 2000)**. The myxoma virus used in developing this GMO was selected for its limited ability to spread in natural rabbit populations **(Barcena *et al* 2000).**

Obviously, such a live virus vaccine is a potential risk to rabbit control in Australia especially if it were to become established in the wild. European countries such as Spain, Portugal and France were equally concerned by former research in Australia into the development of GMOs capable of reducing the fertility of rabbits **(Robinson *et al* 1997).**

Angulo and Barcena (2007) have recently reviewed progress in efforts to gain permission to use the vaccinating virus. The onus is now squarely on Australian biosecurity organizations to undertake a thorough risk analysis.

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