Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV

Ghislaine Le Gall-Reculé [a,*], Françoise Zwingelstein [a], Marie-Philippe Fages [b,c], Stéphane Bertagnoli [b,c], Jacqueline Gelfi [b,c], Jacky Aubineau [d], Alain Roobrouck [d], Giuliana Botti [e], Antonio Lavazza [e], Stéphane Marchandeau [d]

[a] *French Agency for Food, Environmental and Occupational Health Safety (Anses), Laboratory for Studies and Research on Poultry, Pig and Fish Farming (LERAPP), UVIPAC, BP 53, F-22440 Ploufragan, France*
[b] *INRA, UMR1225, F-31076 Toulouse, France*
[c] *Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France*
[d] *National Game and Wildlife Agency (ONCFS), Department of Studies and Research, 39 Bd Albert Einstein, CS 42355, F-44323 Nantes, France*
[e] *The Lombardy and Emilia Romagna Experimental Zooprophylaxis Institute (IZSLER), Department of Virology, via Bianchi 7/9, I-25124 Brescia, Italy*

A B S T R A C T

The existence of non-pathogenic RHDV strains was established when a non-lethal virus named rabbit calicivirus (RCV) was characterised in 1996 in Italy. Since then, different RNA sequences related to RHDV have been detected in apparently healthy domestic and wild rabbits, and recently a new lagovirus was identified in Australia. We have characterised from seropositive healthy domestic rabbits a non-lethal lagovirus that differs from RHDV in terms of pathogenicity, tissue tropism and capsid protein sequence. Phylogenetic analyses have revealed that it is close to the Ashington strain and to the RCV, but distinct. We proved experimentally that it is infectious but non-pathogenic and demonstrated that, contrary to the other described non-pathogenic lagoviruses, it induces antibodies that do not protect against RHDV. Our results indicate the existence of a gradient of cross-protection between circulating strains, from non-protective, partially protective to protective strains, and highlight the extent of diversity within the genus *Lagovirus*.

Keywords:
Calicivirus
Lagovirus
Non-pathogenic virus
RHDV
Phylogeny
Rabbit
Oryctolagus
Cuniculus

Introduction

Rabbit haemorrhagic disease (RHD) is a highly infectious and fatal disease of the European rabbit (*Oryctolagus cuniculus*). It was first described in China (Liu et al., 1984), then subsequently in most parts of the world affecting domestic rabbits and also wild populations. The etiological agent of the disease, the *Rabbit hemorrhagic disease virus* (RHDV), is a non-enveloped single-stranded positive-sense RNA virus belonging to the genus *Lagovirus* (Green et al., 2000) of the family *Caliciviridae* (Meyers et al., 1991; Ohlinger et al., 1990; Parra and Prieto, 1990; Rasschaert et al., 1995).

Several works have serologically evidenced non-pathogenic RHDV-like strains in Europe (Capucci et al., 1997; Chasey et al., 1997; Marchandeau et al., 1998; Rodak et al., 1990; Trout et al., 1997) and also in Australia or New Zealand (Bruce and Twigg, 2004; Bruce et al., 2004; Nagesha et al., 2000; O'Keefe et al., 1999; Parkes et al., 2002; Robinson et al., 2002). The existence of non-pathogenic RHDV strains was established in 1996, when a non-lethal RHDV-like strain genetically distinct from RHDV and called rabbit calicivirus (RCV) was isolated in a rabbitry in Italy (Capucci et al., 1996). This strain differs from the RHDV by its tissue tropism, viral load and the sequence of the gene encoding the capsid protein. Subsequently, apparently non-pathogenic strains were detected after isolation of viral RNA fragments in healthy domestic or wild rabbits from the UK and Ireland (Forrester et al., 2007, 2009; Moss et al., 2002). Lastly, a recent report identified another non-pathogenic lagovirus named RCV-A1 in three apparently healthy young wild Australian rabbits (Strive et al., 2009). The Italian RCV and the Australian RCV-A1 are the only two viruses whose non-lethality has been experimentally confirmed (Capucci et al., 1996; Strive et al., 2010). For all the other strains non-pathogenicity is assumed owing to their isolation in healthy non-vaccinated rabbits. A phylogenetic analysis of RHDV and genetically related non-pathogenic strains based on a fragment of the capsid protein nucleotide sequence showed three distinct genetic groups (Strive et al., 2009). Besides the first group that contains RHDV strains, the second one contains RCV, the Lambay strain from Ireland,

and the Ashington strain from the UK, and the third one contains Australian RCV-A1. These two last groups contain non-pathogenic strains except perhaps the Ashington strain, presumed to be a pathogenic virus although its pathogenicity has not been experimentally proved (Moss et al., 2002). For the non-pathogenic viruses that are genetically related but relatively distant to pathogenic RHDV (i.e., the Italian RCV, the Irish Lambay strain, the English Ashington strain and the Australian RCV-A1) Kerr et al. (2009) proposed the name rabbit calicivirus-like (RCV-like) viruses to distinguish them from RHDV. However, since these viruses are all caliciviruses, it would be better to describe them as non-pathogenic rabbit calicivirus or lagovirus. In addition, a recent study suggested that as RCV and similar strains did not form a monophyletic group with RCV-A1, the name RCV-like was not appropriate for these viruses (Jahnke et al., 2010). We have therefore chosen the name non-pathogenic lagovirus (NP-LV) to designate them.

Competition between non-pathogenic and pathogenic strains and the possible role of the non-pathogenic strains in reducing the impact of RHD have been discussed by several authors on the basis of cross-protection between strains (Boots et al., 2004; Cooke et al., 2000, 2002; Robinson et al., 2002; White et al., 2001). However, the protection conferred by antibodies induced by some NP-LV is lower than that conferred by antibodies induced by virulent RHDV or RCV (Cooke et al., 2002; Nagesha et al., 2000; Strive et al., 2010). Otherwise, a French study based on serological data showed that antibodies produced in response to some NP-LV strains may not protect against RHDV (Marchandeau et al., 2005). These results suggest that study of the competition between strains needs to take into account the existence of non-protective or slightly protective strains, which requires a sufficient knowledge of the diversity of the circulating strains.

In the present work, we have identified a new infectious lagovirus related to RHDV from healthy domestic rabbits and determined the sequence of the gene encoding the VP60 capsid protein in order to establish phylogenetic relationships with other rabbit lagoviruses. In addition, we have investigated its non-lethality for specific pathogen free (SPF) rabbits and the degree of cross-protection induced by the antibodies due to this viral strain against a lethal RHDV challenge.

Results

Serological status of the rabbits

The VP60-RHDV ELISA test performed on sera from 60 healthy 10-week-old rabbits from the rabbitry revealed that 38% of them were seropositive at D0 and 88% 1 week later, indicating that an active infection was in progress in the rabbitry (Table 1). Sera from 24 rabbits seropositive at D6 and sacrificed for virological screening at D13 were tested with the cELISA and anti-isotype ELISA tests specific to RHDV. None were positive by cELISA, IgM, or IgA ELISA (<10), but some of them showed low levels of IgG (40–160) (Table 1). According to Cooke et al. (2000), these last rabbits (cELISA titres ≤ 10, with moderate IgG antibodies but without IgA or IgM) were considered to have been infected with a non-pathogenic lagovirus. Therefore, the antibodies detected with the VP60-RHDV ELISA test were not specific to pathogenic RHDV.

Sequence analysis of the capsid protein

Duodenum samples gave the first positive RT-PCR results when liver or spleen samples gave negative results. We therefore focused on duodenum samples to continue the search for viral RNA. Nineteen of the 29 duodenum intestines samples tested were positive and PCR product sequencing results confirmed that they were close to rabbit lagovirus sequences. Among the PCR-positive samples, sample JA1641 (strain 06-11) was subjected to a full-length VP60 PCR amplification and the PCR product was sequenced.

Table 1
Antibody responses in the 60 rabbits used in this study.

Rabbit*	Serology D0	Serology D6					Serology D13
	VP60-ELISA†	VP60-ELISA	cELISA	IgG	IgA	IgM	VP60-ELISA
AR02	+	++++					
AR04	++	++++					
AR06‡	−	++++	<10	80	<10	<10	
AR08‡	++	++	<10	40	<10	<10	
AR10	++	++					
AR12‡	−	+++	<10	40	<10	<10	
AR14	++	++++					
AR16	++	++++					
AR18	+++	++++					
AR20	+++	++++					
AR26‡	−	++++	<10	40	<10	<10	
AR28‡	−	+	<10	40	<10	<10	
AR30	++	++++					
AR32‡	−	++++	<10	80	<10	<10	
AR34	−	++++					
AR36‡	−	++++	<10	40	<10	<10	
AR38‡	−	+++	<10	40	<10	<10	
AR40‡	−	++++	<10	40	<10	<10	
AR42‡	−	++++	<10	<10	<10	<10	
AR44	+	++++					
AR46	−	++++					
AR48‡	−	+++	<10	40	<10	<10	
AR50	−	++					
AR52	+++	++++					
AR54‡	−	+++	<10	<10	<10	<10	
AR56	−	++++					
AR58‡	−	++++	<10	<10	<10	<10	
AR60	−	++++					
AR62‡	−	++++	<10	160	<10	<10	
AR64	−	++++					
JA1611§	−	-					−
JA1613‡	+	+++	<10	<10	<10	<10	
JA1615‡	−	+++	<10	<10	<10	<10	
JA1617	−	−					+
JA1619	++	++					
JA1621	−	−					++
JA1623	−	−					++
JA1625	−	+					+
JA1627	+++	++++					
JA1629	++	++++					
JA1631	−	+					++
JA1633	++	++					
JA1635§	−	+					+
JA1637§	−	−					−
JA1639	++	++++					
JA1641§	−	−					−
JA1651‡	−	++++	<10	<10	<10	<10	
JA1653‡	−	++++	<10	<10	<10	<10	
JA1655	++	++++					
JA1657‡	+	+++	<10	<10	<10	<10	
JA1659	++	++++					
JA1663	++	++++					
JA1665‡	−	+++	<10	<10	<10	<10	
JA1667‡	−	++	<10	<10	<10	<10	
JA1669‡	−	+++	<10	<10	<10	<10	
JA1671§	−	−					−
JA1684	++	++++					
JA1686	+	++++					
JA1688‡	−	+++	<10	80	<10	<10	
JA1690‡	−	+++++	<10	<10	<10	<10	

*The rabbit number corresponds to the location where the rabbit was placed in quarantine ("AR" or "JA") followed by an order number. Numbers in italics and in bold correspond to the rabbits used for the experimental study of protection against RHDV challenge (five rabbits). A number in italics, in bold and underlined corresponds to the rabbits remained in the "JA" location that died from a natural RHD between D19 and D22 (9 rabbits). The number in a grey box (JA1641) corresponds to the rabbit from which the strain 06-11 was identified and characterised.
†Serological status determined using VP60-RHDV ELISA. The scoring system is described in materials and methods section.
‡The serum was also screened with competitive ELISA (cELISA) and anti-isotype ELISA tests (Capucci et al., 1997; Cooke et al., 2000; Lavazza and Capucci, 2008a). <10: negative; 40–160: positive with a low titre. The corresponding rabbits were sacrificed at D13 for virological analysis (24 rabbits).
§ The rabbit were sacrificed at D21 for virological analysis (5 rabbits).

The 06-11 VP60 gene is 1734 nucleotides (nt) long (578 amino acids long) and when compared to RHDV, has a 6 nt deletion in length at position 921–927 which corresponds to the amino acids (aa) 308 and 309 of the VP60 protein of RHDV. A similar deletion is present at the same location in the Ashington strain genome and at aa 309 and 310 in the RCV genome. The average nucleotide identity between the VP60 gene sequences of 06-11 and RHDV is 83%. The 06-11 strain shared 85% identity with RCV but only 79% with the non-pathogenic RCV-A1. In contrast, this identity reached 93.8% with the Ashington sequence available in nucleotide databases (1542 nt/1644 nt identity).

The 06-11 VP60 deduced amino acid sequence was aligned with those of some RHDV reference strains including one antigenic variant RHDVa strain, RCV, Ashington and RCV-A1 (Fig. 1). The average amino acid similarity between 06-11 and RHDV is 90.8%. It is 92.9% with RCV, 87% with RCV-A1 and 97.8% with the Ashington sequence. The majority of the 06-11 substitutions (83%) are located in the most variable part of the capsid protein, the C-terminal half. When we compared the sequences within the two regions that show the highest degree of genetic variation (region C from aa 301 to 310 and region E from aa 344 to 434, according to the nomenclature of Neill (1992)), similarities decreased to about 81.2%, 79.2%, or 63.4%, between 06-11 and RHDV, RCV, or RCV-A1, respectively. These data emphasised the differences between the viruses. On the contrary, the similarity between the 06-11 and Ashington sequences reached 95%, and even 100% within the hypervariable region C which also contains the two amino acid deletions.

Genetic relationship

Irrespective of the method used, phylogenetic analyses implemented with the almost entire capsid protein nucleotide sequences of RCV-A1, 32 RHDV and RCV isolates gave similar results. They revealed that the rabbit lagoviruses were clustered into three major genetic groups, (i) a group comprised of the pathogenic RHDV strains further separated into viruses related to the prototype RHDV and the antigenic variant RHDVa, (ii) a group comprised of the RCV strain together with the Ashington strain and the 06-11 strain described in this study, and (iii) a group comprised of RCV-A1 strain alone (Fig. 2). We noticed that the RCV, Ashington and 06-11 viruses showed significant genetic variation from each other and from the RHDV sequences.

Experimental evidence of a replicating non-lethal lagovirus

An experimental study was performed to test whether the 06-11 strain could be transmitted by inoculation or through contact from NP-LV infected rabbits to serologically naïve SPF rabbits without causing disease. Serological analyses using the VP60-RHDV ELISA test confirmed that, at the beginning of the assay, the two SPF rabbits were seronegative and the three domestic rabbits were seropositive with low to moderate titres. The presence of antibodies was detected in the blood sample collected as early as D5 from the SPF rabbit in close contact with the three domestic rabbits (low titre) and in the blood sample collected at D10 from the inoculated SPF rabbit (moderate titre). No sign of RHD was detected during the assay, and at necropsy no lesions were seen. Screening for NP-LV RNA was performed in duodenum samples collected from the five rabbits at the sacrifice. We succeeded in amplifying a DNA fragment of the expected size for all the rabbits (PCR U38-L337). The VP60 sequence determined from the SPF rabbit samples was identical to the sequence of the NP-LV strain 06-11 previously established. Thus, we confirmed that this virus can be experimentally transmitted to SPF rabbits without causing clinical signs or mortality.

Field and experimental evidences of a lagovirus that induces non-protective antibodies

In "JA" location, a natural RHD outbreak occurred at D19 in one hutch where 10 seropositive rabbits, showing moderate to very high antibody levels at D6 (Table 1), were kept and fed with grass taken from a RHDV-contaminated area. Nine rabbits died within 4 days. At necropsy typical RHD lesions were observed and all the sampled livers were RHDV-positive by RT-PCR test.

In order to experimentally determine whether the NP-LV antibodies detected by the VP60-RHDV ELISA test conferred cross-protection to rabbits against subsequent infection with RHDV, an infection study was performed on five seropositive rabbits with very high antibody levels (Table 1). Three rabbits were challenged with an intramuscular inoculation of a dose of RHDV commonly used to check the efficacy of commercial vaccines and died 2–4 days post-infection. The two non-inoculated rabbits placed in contact in an adjacent cage died 4–6 days post-infection. These rates and timeframes of mortality for inoculated or contact animals are consistent with the expected results following the standard viral challenge on immunologically naïve rabbits. By comparison, vaccinated rabbits exhibiting even low RHDV antibody levels as determined with VP60-RHDV ELISA were shown to be protected against the challenge dose used in this study (Bertagnoli et al., 1996). At necropsy typical RHD lesions were observed and all the sampled livers were RHDV-positive by RT-PCR test.

Discussion

The observation of a spontaneous serological conversion of rabbits more than 2 months old, i.e., without maternal antibodies and having become sensitive to RHD, showed that an active infection was in progress in the rabbitry. The absence of associated mortality suggested that a non-pathogenic virus serologically related to RHDV was responsible for these seroconversions. A similar overall clinical picture previously led to the characterisation of the RCV in an Italian industrial rabbitry (Capucci et al., 1996). In the present study, experimental investigation showed that the virus is highly infectious as described for RHDV, but non-lethal for rabbits. We could not assume that it is definitively a non-pathogenic virus since we did not histologically examined the representative organs of died rabbits. Thus, we cannot exclude that histological changes may be evident even in the absence of macroscopic changes. Future histological studies will be necessary to clearly demonstrate the lack of pathogenicity of this virus. However, the name of non-pathogenic has been commonly used to characterise the non-lethal lagoviruses isolated in healthy rabbits such as RCV or RCV-A1 even when no histological studies have been performed.

We also confirmed that, in contrast to the RCV or RCV-A1, the antibodies raised against this strain irrespective of their titre do not protect against RHDV. Indeed, a total of 14 out 15 seropositive rabbits died from RHD, including the nine remained in one of the quarantine locations which died following a natural RHDV infection. In a previous study conducted during a RHDV outbreak in a free-living population of rabbits, we observed that some rabbits died from RHD even if they were seropositive for anti-RHDV antibodies and had expected protective titres (Marchandeau et al., 2005). Some recent molecular data proving the circulation of closely related NP-LV strains in French wild rabbit populations (unpublished results) are in agreement with the present results. Indeed, it is confirmed the hypothesis initially proposed, i.e., these detectable but non-protective antibodies were induced by an NP-LV infection.

We have identified a lagovirus (06-11 strain), which differs from the RHDV strains in terms of pathogenicity, but also in terms of tissue tropism (small intestine) and sequence of the VP60 capsid protein. These characteristics are consistent with findings on RCV and RCV-A1.

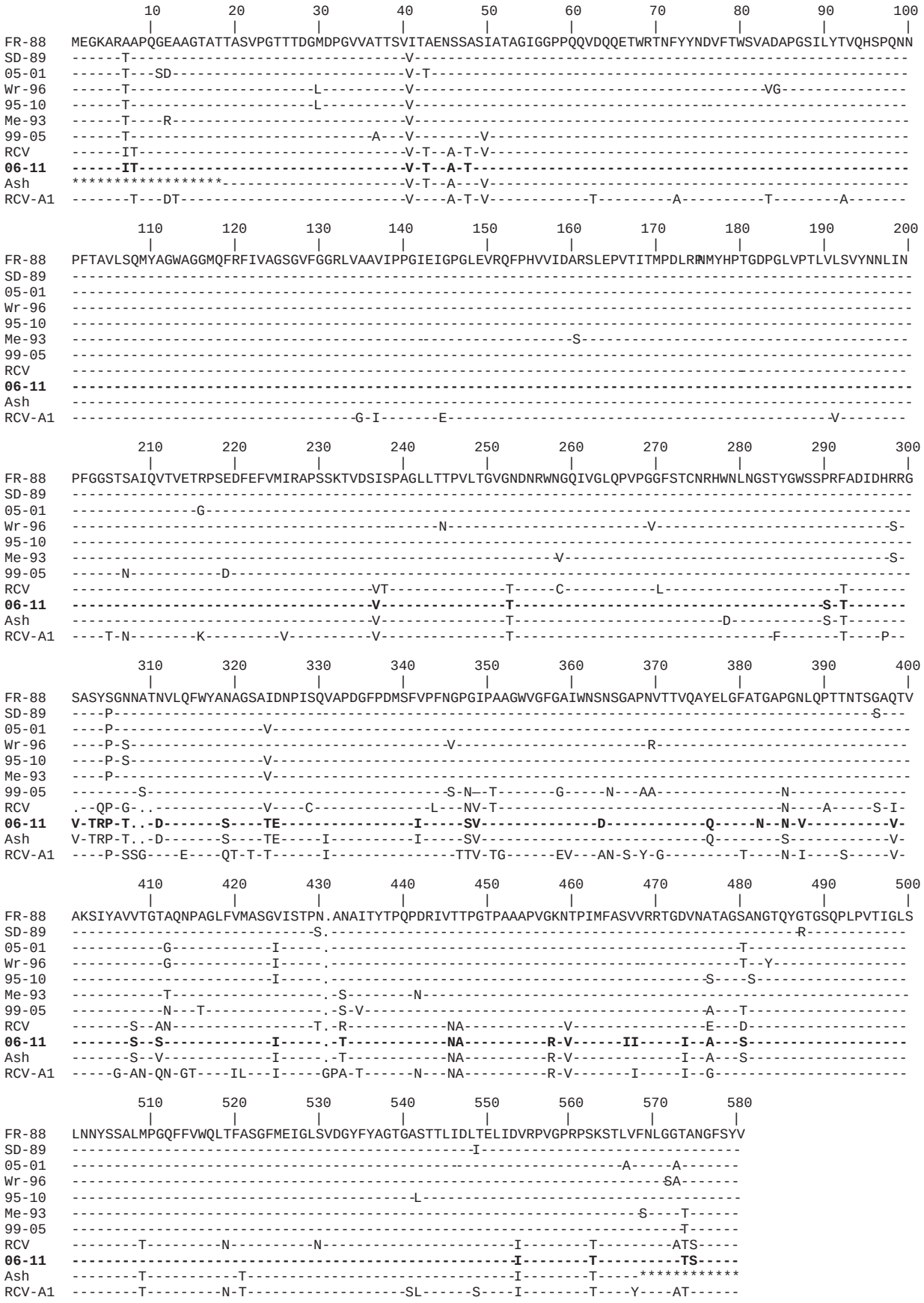

Fig. 1. Amino acid sequence alignment of the VP60 capsid protein from seven representative pathogenic RHDV strains including the antigenic variant RHDVa, the non-pathogenic Italian RCV and Australian RCV-A1 strains, the UK Ashington strain and the 06-11 strain characterised in this study. The strain names in a shortened form are explained in Table 2.

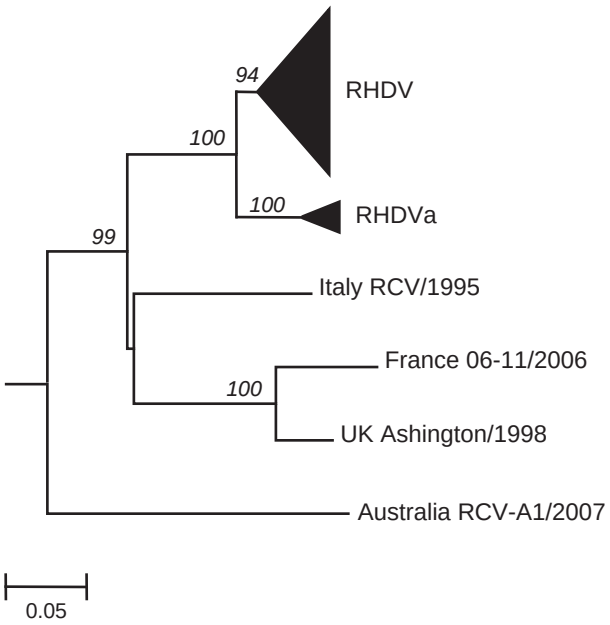

Fig. 2. Phylogenetic tree derived for nucleotide sequences of 27 RHDV and 5 antigenic variant RHDVa strains (detailed in Table 2), the non-pathogenic Italian RCV and Australian RCV-A1 strains, the UK Ashington strain and the NP-LV 06-11 strain, using the minimum evolution method. Bootstrap values greater than 70% (for 1000 replicates) are given in italics after each node. The French reference *European brown hare syndrome virus* strain EBHSV-GD (accession number Z69620) was used as an outgroup to root the tree. The RHDV and RHDVa branches are collapsed to highlight the main genogroups.

Regarding the VP60 gene sequence, 06-11 is closer to the RCV than to RHDV or RCV-A1, but the highest nucleotide and amino acid identity was with the Ashington strain. The majority of the 06-11 amino acid substitutions are found in the C-terminal half of the VP60 protein which forms the outer shell of the capsid of the caliciviruses (Laurent et al., 1994; Neill, 1992; Prasad et al., 1994). This part of the RHDV capsid contains the main antigenic regions (Barcena et al., 2004; Capucci et al., 1995; Capucci et al., 1998; Martinez-Torrecuadrada et al., 1998; Meyers et al., 1991). Within this part, the P2 subdomain which forms an external loop and includes the regions C and E defined by Neill (1992) contains determinants for cell binding, host specificity and strain diversity (Chen et al., 2004; Tan et al., 2006). The relatively low sequence similarity between 06-11 and pathogenic RHDV strains in the P2 subdomain might explain the differences in tropism and lethality. In addition, following the characterisation of RCV, Lavazza and Capucci (2008b) suggested that the region C of the capsid for RHDV could influence the degree of pathogenicity of the caliciviruses. The description for the first time of a strain that was demonstrated to be non-pathogenic but also to have significant sequence variation within the region C could reinforce the hypothesis that this region takes part in viral pathogenicity. In other respects, Esteves et al. (2008) and more recently Kinnear and Linde (2010) showed a positive selection that occurs in the outer shell of the RHDV VP60 protein and drives amino acid substitutions at some specific sites (named positively selected codons or PSCs) within regions where the main antigenic determinants were described. These authors therefore suggested that variation of a PSC is likely to be related to pathogenicity and virulence. Amino acid differences observed in the 06-11 sequence at some of the identified PSCs (aa 307 and 432), and notably in the region C of the capsid, seems to confirm their importance in the antigenic diversity within the *Caliciviridae*.

The 06-11 strain represents a new NP-LV clustered into the Ashington lineage and close to RCV. The Ashington strain was supposed to be pathogenic according to Moss et al. (2002) but it has never been implicated in any other recognisable epidemic outbreak in the UK (Forrester et al., 2009). Its close relationship with the 06-11 strain could suggest that this strain is non-lethal and experimental confirmation or invalidation of its pathogenicity would be very useful to understand the genetic evolution of these two viruses. Indeed, the phylogenetic tree topology suggested that the RCV and the 06-11/Ashington lineage emerged long before the divergence of the pathogenic RHDV lineage, with the RCV emerging prior to the 06-11/Ashington lineage in agreement with previous published results (Kerr et al., 2009; Moss et al., 2002). In addition, our results showed a significant genetic variation between 06-11 and Ashington indicating that these two strains may possibly have evolved independently. Regarding the two other divergent Lambay and Pit-WD strains described in the UK (Forrester et al., 2007, 2009), although only one third of their VP60 sequence is known, results also showed a high genetic variation between 06-11 and these two strains, especially with the latter (only 85.6% amino acid similarity). All these data indicate considerable divergence between these different NP-LV strains. The mean time to the most recent common ancestor (TMRCA) of RHDV and NP-LV strains was estimated to be over 80 (Kinnear and Linde, 2010) or 200 years ago (Kerr et al., 2009). In the only study that includes the Ashington strain in its analyses, the mean TMRCAs of the Ashington and Australian RCV-A1 strains fall in the 19th century (Kerr et al., 2009). The authors correlated this time with the date of introduction of domestic European rabbits to Australia and the resulting presence of RCV-A1. However, our phylogenetic results show that the RCV-A1 lineage diverged long prior to the 06-11/Ashington one. It would be interesting to carry on screening for NP-LV strains in European countries to identify a virus closer to RCV-A1, which would be more likely to be at the source of the Australian virus.

These data confirm the circulation of several different non-pathogenic lineages and highlight the extent of diversity within the lagovirus genus. Moreover, among this high diversity, our results show the existence of a gradient of cross-protection between strains. Indeed, whereas we have demonstrated the existence of NP-LV strains, previous studies have shown partial cross-protection between RHDV and RCV-A1 viruses, low titres being non-protective (Cooke et al., 2002; McPhee et al., 2009; Strive et al., 2010). Cross-protection is complete with RCV (Capucci et al., 1996) or between RHDV viruses as shown by the efficacy of vaccine strains. To better understand RHDV epidemiology in wild populations, besides taking into account factors such as population size and climate (Cooke et al., 2002; Mutze et al., 1998; Parkes et al., 2002), host genetics (Guillon et al., 2009), and virulence of viral strains (Fouchet et al., 2009), we need to determine to what extent NP-LV strains fail to induce protective immunity against RHDV. Indeed, differences of cross-protection might explain the described variability of the impact of RHD on rabbit populations. The existence of non-protective strains as well as the evidence of recombination between strains (Abrantes et al., 2008; Forrester et al., 2008) should lead to reconsideration of the interaction between pathogenic and non-pathogenic strains, which is likely to be more complex than previously assumed. Studies are underway to identify the full-length genome sequence of 06-11 strain. Complete molecular data on a non-pathogenic and non-protective lagovirus should enable understanding of RHDV evolution through a comparison with data already available on some RHDVs (Forrester et al., 2008) and on the evolutionarily earlier RCV-A1 lineage (Strive et al., 2009).

Materials and methods

Animals

Sixty 10-week-old New Zealand White rabbits showing no apparent sign of disease were placed in quarantine into several hutches in two distinct locations of several kilometres far away each other ("AR" and "JA"; Table 1) before being used in an experimental

assay in the field as sentinel animals. The rabbits were raised without vaccination against RHDV or myxomatosis in a controlled small rabbitry which had maintained good sanitary conditions for several years. To determine their serological status against RHDV, sera were collected onto blotting paper twice, 1 week apart, at D0 and at D6, and blood samples were screened for the presence of RHDV antibodies. Sera from the seronegative or seropositive with low titre rabbits at D6 were collected again at D13.

To attempt to identify NP-LV viruses, we sacrificed rabbits at the beginning of the seroconversion to collect liver, spleen and small intestine (duodenum) samples. We therefore sacrificed at D13 24 rabbits among those that were seronegative or seropositive with low or moderate antibody titres at D0, and sacrificed at D21 five rabbits that were seronegative or having low titres at D6. The other 31 rabbits were kept in the hutches.

Antibody detection

A VP60-RHDV ELISA based on the detection of a baculovirus-expressed capsid protein (VP60) antigen that self-assembles into virus-like particles (Laurent et al., 1994) was used for semi-quantitative measurement of the antibody titres according to the method described in Marchandeau et al. (2005). A serum was considered to be negative when the ratio sample OD_{405} (optical density)/negative control OD_{405} was below 3, positive with low titre (+) when the ratio was between 3 and 4, positive with moderate titre (++) when the ratio was between 4 and 6, positive with high titre (+++) when the ratio was between 6 and 8, and positive with very high titre (++++) when the ratio was above 8. As this ELISA does not enable discrimination of antibodies due to RHDV infection from those due to RHDV-like infections (Marchandeau et al., 2005), some positive sera ($n=24$) were also screened with a competitive ELISA (cELISA) and anti-isotype ELISA tests (Capucci et al., 1997) as described in Cooke et al. (2000, 2002) and Lavazza and Capucci (2008a). The cELISA recognises an external epitope specific to RHDV (Capucci et al., 1997). This test using a monoclonal antibody is considered as the standard, reference serological test for RHD (Lavazza and Capucci, 2008a). The anti-isotype ELISA tests detect IgM, IgG and IgA specific to RHDV. They are used to interpret field serology and to determine the immunological status of rabbits (Lavazza and Capucci, 2008b; Cooke et al., 2002). The use of these different tests makes it possible to detect a large range of antibodies raised against rabbit lagoviruses and to discriminate antibodies raised against RHDV from those raised against other rabbit lagoviruses.

RT-PCR and sequence analysis

Each frozen liver sample was thawed and 100 µl of the obtained exudate was collected. For the other tissue samples (duodenum and spleen), 30 mg was homogenised using a mixer-mill disruptor (TissueLyser, QIAGEN). RNAs were extracted using the RNeasy Mini kit (QIAGEN) and viral RNAs were reverse transcribed using oligo-dT (Invitrogen) as a primer and SuperScript™ II Reverse Transcriptase (Invitrogen).

For the initial screening, several pairs of RHDV primers for polymerase chain reaction (PCR) amplification were used, among which were three pairs previously defined to amplify overlapping genomic regions of the gene encoding the VP60 (Le Gall-Reculé et al., 2003). Two successive amplifications with the same PCR primers using the AmpliTaq Gold® DNA polymerase (Applera Applied Biosystems) were necessary to obtain sufficient quantities of DNA to allow their sequencing. The amplified PCR products were visualised by electrophoresis on agarose gel and purified (UltraClean™ 15 DNA Purification Kit, Mo Bio Laboratories) prior to sequencing. The DNA sequence was determined twice in both directions by the dye terminator method (ABI PRISM DyeTerminator Cycle Sequencing Ready Reaction kit, Applera Applied Biosystems) on an ABI 373XL automatic DNA sequencer (Applera Applied Biosystems) with the PCR primers and sequencing primers (Le Gall-Reculé et al., 2003). To confirm the assembled VP60 gene sequence, positive cDNAs were subjected to one run of full-length VP60 PCR amplification using a specific primer pair designed from the preliminary NP-LV VP60 gene sequences we obtained. A high-fidelity DNA polymerase (Expand High Fidelity^{PLUS} PCR System, Roche Applied Science) was used. The DNA sequence was determined using PCR and sequencing primers as described above (primer sequences available upon request). The deduced amino acid sequences of the VP60 gene were obtained using the software available on the Biosupport website. Multiple amino acid sequence alignments were generated by the CLUSTALW method using the NPS website.

Following RHDV infections, RNA was extracted from liver exudates using a commercial kit (RNeasy Mini kit, QIAGEN) and tested for RHDV RNA by RT-PCR as described by Le Gall-Reculé et al. (2003). Screening for NP-LV RNA was performed from duodenum samples as described in the previous paragraph, except that RNA was extracted in a preliminary step with TRIzol® Reagent (Invitrogen) before the use of "RNeasy" columns to increase the sensitivity. For the screening of positive samples, we used a pair of PCR primers ("U38" 5' CAGCGGGCACTGCTACCACAGCATC 3' and "L337" 5' GAAGCGAAACTG-CATGCCACCRGCCCA 3') derived from the one (Rab1b-Rab2) defined by Tanja Strive (CSIRO, Australia) who kindly provided us with the sequences before their publication (Strive et al., 2009). Then, the full-length VP60 PCR amplification was performed.

Phylogenetic analyses

Phylogenetic relationships were inferred using entire sequences of the capsid protein VP60 gene available in databases from some representative pathogenic RHDV and the antigenic variant RHDVa (Capucci et al., 1998), the non-pathogenic Italian RCV strain and the non-pathogenic lagovirus RCV-A1 recently characterised in Australia. We also included the sequence of the divergent Ashington strain from the UK since it is almost complete (95% of the gene). The accession number, country of origin and year of collection of the 36 sequences are listed in Table 2. The sequence of the French reference *European brown hare syndrome virus* (EBHSV) strain EBHSV-GD (accession number: Z69620 (Le Gall et al., 1996)) was used as an outgroup to root the trees. Phylogenetic analyses were conducted using MEGA software version 3.1 (Kumar et al., 2004) for phenetic and cladistic characterisation. Multiple sequence alignments were generated by the CLUSTALW method. For the phenetic analysis, the Minimum Evolution (ME) method was implemented with the Kimura 2-parameter model including transition and translation substitutions. The search for the ME tree was implemented with the close-neighbour-interchange option and the Neighbour-joining algorithm was used to generate the initial tree. For the cladistic method, Maximum Parsimony (MP) was used and the search for the MP tree was implemented with the close-neighbour-interchange algorithm. For the two methods, codon positions included were the 1st and the 2nd, and the pairwise deletion option was selected. The topology of trees was evaluated by 1000 bootstrap analyses. Majority-rule consensus trees were determined by the CONSENSE program and were drawn using MEGA software version 3.1.

Experimental studies of viral transmission and of protection against RHDV infection

Two experimental studies were performed under negative pressure in rooms at BSL2 experimental facilities with filtered air according to biosafety and bioethical procedures, under the authorization of animal experimentation number 22–24 delivered by the

Table 2
Sequences of RHDV, antigenic variant RHDVa (in italics), RCV, RCV-A1, Ashington and 06-11 strains used for phylogenetic analyses. The country of origin, the name of the isolate and the year of collection are included in the name when known. The sequence names are ordered according to their position in the phylogenetic tree.

Name: country isolate/year of collection (shortened names in Fig. 1)	GenBank/EMBL accession number
UK Rainham/1993	AJ006019
France 95-10/1995 (95-10)	AJ535094
Germany Frankfurt/1996	Y15424
Italy BS/1989	X87607
Germany Meiningen/1993 (Me-93)	Y15426
Germany Hagenow/1990	Y15441
UK Ascot/1992	EF558575
France 95-05/1995	AJ535092
France 00-13/2000	AJ495856
France 05-01/2005 (05-01)	AM085133
Barhain/2001	DQ189077
Germany Wriezen/1996 (Wr-96)	Y15427
UK Ireland12/2001	AY926883
Saudi Arabia/1996	DQ189078
China WX/1984	AF402614
France Haute-Saone/1988 (FR-88)	U49726
Mexico/1989	AF295785
Korea/1990	EU003580
Italy/1990	EU003579
Germany FRG/1989	M67473
New Zealand,NZ54/2003	EF558579
New Zealand	AF231353
Czech V351/1987	U54983
France 00-08/2000	AJ329594
Germany Eisenhuettenstadt/1989	Y15440
Spain AST/1989	Z49271
France SD/1989 (SD-89)	Z29514
Germany Triptis/1996	Y15442
China CD/2004	AY523410
USA Iowa/2000	AF258618
France 99-05/1999 (99-05)	AJ302016
France 03-24/2003	AJ969628
Italy RCV/1995 (RCV)	X96868
France 06-11/2006 (06-11)	AM268419
UK Ashington/1998 (Ash)	EF558587
Australia RCV-A1/2007 (RCV-A1)	EU871528

local director of veterinary services on behalf of the prefect of Côtes d'Armor department.

First, in order to study the transmission route and lethality of the 06-11 strain, two 10-week-old specific-pathogen-free (SPF) commercial New Zealand White rabbits were used. To test the lethality of the virus isolated in the rabbitry, one SPF rabbit was inoculated by the oro-nasal route with filtered (0.22 μm) supernatant of a pool of duodenum samples from which RHDV-like RNAs had been characterised. In order to study the possibility of viral transmission through contact, the other SPF rabbit was placed in a cage with three 9-week-old healthy rabbits taken from the rabbitry. Blood samples were collected onto blotting paper from the five rabbits at the beginning of the assay (D0), then twice 5 days apart (D5 and D10) and at the sacrifice (D14). Detection of antibodies was performed using the VP60-RHDV ELISA test. Daily observations for morbidity (asthenia, anorexia, respiratory and nervous signs) and mortality were performed. Surviving animals were killed humanely and examined for macroscopic lesions. Duodenum and liver samples were collected for virological analyses.

Second, in order to determine whether the detected 06-11 antibodies conferred cross-protection to rabbits against subsequent RHDV infection, five rabbits exhibiting very high titres of antibodies were selected for an experimental challenge (Table 1). They were split into two groups: (i) three rabbits were challenged with a standardised intramuscular inoculation of 10^3 LD_{50} of RHDV reference strain isolated in 1988 in France and (ii) two rabbits were placed in an open wire-meshed cage adjacent to that of inoculated rabbits that allowed direct contact between the rabbits to favour infection by a natural route. Daily observations for morbidity (asthenia, anorexia, respiratory and nervous signs) and mortality were performed. Dead rabbits were examined for macroscopic lesions and liver samples were collected for virological analyses.

Nucleotide sequence accession number

The nucleotide sequence of the NP-LV strain 06-11 is available in databases under the GenBank/EMBL accession number AM268419.

Acknowledgments

This work was supported by the ANR SEST program "Pathocénose et émergence des maladies transmissibles" [Pathocenosis and emergence of transmissible diseases] and the Fédération Nationale des Chasseurs. The authors would like to acknowledge the personnel of the breeding and experimentation in avian pathology service (Anses, LERAPP, France) and the avian and rabbit breeding service (Anses, LERAPP, France), as well as Brigitte Peralta and Josyane Loupias (ENVT, UMR 1225, France) for their excellent technical assistance. We are also very grateful to Tanja Strive (CSIRO, Australia) for providing sequences of two PCR primers and Lorenzo Capucci (IZSLER, Italy) for critically reviewing this manuscript.

References

Abrantes, J., Esteves, P.J., van der Loo, W., 2008. Evidence for recombination in the major capsid gene VP60 of the rabbit haemorrhagic disease virus (RHDV). Arch. Virol. 153, 329–335.
Barcena, J., Verdaguer, N., Roca, R., Morales, M., Angulo, I., Risco, C., Carrascosa, J.L., Torres, J.M., Caston, J.R., 2004. The coat protein of rabbit hemorrhagic disease virus contains a molecular switch at the N-terminal region facing the inner surface of the capsid. Virology 322, 118–134.
Bertagnoli, S., Gelfi, J., Le Gall, G., Boilletot, E., Vautherot, J.-F., Rasschaert, D., Laurent, S., Petit, F., Boucraut-Baralon, C., Milon, A., 1996. Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit viral hemorrhagic disease virus capsid protein. J. Virol. 70, 5061–5066.
Boots, M., Hudson, P.J., Sasaki, A., 2004. Large shifts in pathogen virulence relate to host population structure. Science 303, 842–844.
Bruce, J.S., Twigg, L.E., 2004. Rabbit haemorrhagic disease virus: serological evidence of a non-virulent RHDV-like virus in south-western Australia. Wildl. Res. 31, 605–612.
Bruce, J.S., Twigg, L.E., Gray, G.S., 2004. The epidemiology of rabbit haemorrhagic disease, and its impact on rabbit populations, in south-western Australia. Wildl. Res. 31, 31–49.
Capucci, L., Frigoli, G., Ronshold, L., Lavazza, A., Brocchi, E., Rossi, C., 1995. Antigenicity of the rabbit hemorrhagic disease virus studied by its reactivity with monoclonal antibodies. Virus Res. 37, 221–238.
Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit haemorrhagic disease virus but non pathogenic. J. Virol. 70, 8614–8623.
Capucci, L., Nardin, A., Lavazza, A., 1997. Seroconversion in an industrial unit of rabbits infected with a non-pathogneic rabbit haemorrhagic disease-like virus. Vet. Rec. 140, 647–650.
Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L., Brocchi, E., 1998. A further step in the evolution of rabbit haemorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Res. 58, 115–126.
Chasey, D., Trout, R.C., Edwards, S., 1997. Susceptibility of wild rabbits (Oryctolagus cuniculus) in the United Kingdom to rabbit haemorrhagic disease (RHD). Vet. Res. 28, 271–276.
Chen, R., Neill, J.D., Noel, J.S., Hutson, A.M., Glass, R.I., Estes, M.K., Prasad, B.V., 2004. Inter- and intragenus structural variations in caliciviruses and their functional implications. J. Virol. 78, 6469–6479.
Cooke, B.D., Robinson, A.J., Merchant, J.C., Nardin, A., Capucci, L., 2000. Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiol. Infect. 124, 563–576.
Cooke, B.D., McPhee, S., Robinson, A.J., Capucci, L., 2002. Rabbit haemorrhagic disease: does a pre-existing RHDV-like virus reduce the effectiveness of RHD as a biological control in Australia? Wildl. Res. 29, 673–682.
Esteves, P.J., Abrantes, J., Carneiro, M., Müller, A., Thompson, G., van der Loo, W., 2008. Detection of positive selection in the major capsid protein VP60 of the rabbit haemorrhagic disease virus (RHDV). Virus Res. 137, 253–256.
Forrester, N.L., Trout, R.C., Gould, E.A., 2007. Benign circulation of Rabbit haemorrhagic disease virus on Lambay Island, Eire. Virology 358, 18–22.
Forrester, N.L., Moss, S.R., Turner, S.L., Schirrmeier, H., Gould, E.A., 2008. Recombination in rabbit haemorrhagic disease virus: possible impact on evolution and epidemiology. Virology 376, 390–396.

Forrester, N.L., Boag, B., Buckley, A., Moureau, G., Gould, E.A., 2009. Co-circulation of widely disparate strains of rabbit haemorrhagic disease virus could explain localised epidemicity in the United Kingdom. Virology 393, 42–48.
Fouchet, D., Le Pendu, J., Guitton, J.-S., Guiserix, M., Marchandeau, S., Pontier, D., 2009. Evolution of microparasites in spatially and genetically structured host populations: the example of RHDV infecting rabbits. J. Theor. Biol. 257, 212–227.
Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J., Thiel, H.J., 2000. Taxonomy of the caliciviruses. J. Infect. Dis. 181 (Suppl 2), S322–S330.
Guillon, P., Ruvoen-Clouet, N., Le Moullac-Vaidye, B., Marchandeau, S., Le Pendu, J., 2009. Association between expression of the H histo-blood group antigen, α1, 2fucosyltransferases polymorphism of wild rabbits, and sensitivity to rabbit hemorrhagic disease virus. Glycobiology 19, 21–28.
Jahnke, M., Holmes, E.C., Kerr, P.J., Wright, J.D., Strive, T., 2010. Evolution and phylogeography of the non-pathogenic calicivirus RCVC-A1 in wild rabbits in Australia. J. Virol. 94, 12397–12404.
Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. J. Virol. 83, 12129–12138.
Kinnear, M., Linde, C.C., 2010. Capsid divergence in rabbit hemorrhagic disease virus. J. Gen. Virol. 91, 174–181.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.
Laurent, S., Vautherot, J.-F., Madelaine, M.-F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into virus-like particles and induces protection. J. Virol. 68, 6794–6798.
Lavazza, A., Capucci, L., 2008a. Rabbit haemorrhagic disease, Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), sixth ed. O.I.E, Paris, pp. 947–961.
Lavazza, A., Capucci, L., 2008b. How many caliciviruses are there in rabbits? A review on RHDV and correlated viruses. In: Alves, P.C., Ferrand, N., Hackländer, K. (Eds.), Lagomorph biology: evolution, ecology, and conservation. Springer-Verlag, Berlin Heildelberg, pp. 263–278.
Le Gall, G., Huguet, S., Vende, P., Vautherot, J.-F., Rasschaert, D., 1996. European brown hare syndrome virus: molecular cloning and sequencing of the genome. J. Gen. Virol. 77, 1693–1697.
Le Gall-Reculé, G., Zwingelstein, F., Laurent, S., de Boisseson, C., Portejoie, Y., Rasschaert, D., 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants. Arch. Virol. 148, 65–81.
Liu, S.J., Xue, X.P., Pu, B.Q., Quian, N.H., 1984. A new viral disease in rabbits [in Chinese]. Anim. Husb. Vet. Med. 16, 253–255.
Marchandeau, S., Ricci, J.-C., Chantal, J., 1998. Serological prevalence rate of the virus of the Rabbit Haemorrhagic Disease and its related forms in different wild populations [in French]. Mammalia 62, 95–103.
Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineau, J., Botti, G., Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Vet. Res. 36, 53–62.
Martinez-Torrecuadrada, J.L., Cortes, E., Vela, C., Langeveld, J.P., Meloen, R.H., Dalsgaard, K., Hamilton, W.D., Casal, J.L., 1998. Antigenic structure of the capsid protein of rabbit haemorrhagic disease virus. J. Gen. Virol. 79, 1901–1909.
McPhee, S.R., Butler, K.L., Kovaliski, J., Mutze, G., Capucci, L., Cooke, B.D., 2009. Antibody status and survival of Australian wild rabbits challenged with rabbit haemorrhagic disease virus. Wildl. Res. 36, 447–456.
Meyers, G., Wirblich, C., Thiel, H.J., 1991. Rabbit hemorrhagic disease virus -molecular cloning and nucleotide sequencing of a calicivirus genome. Virology 184, 664–676.
Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of Rabbit haemorrhagic disease virus. J. Gen. Virol. 83, 2461–2467.
Mutze, G., Cooke, B., Alexander, P., 1998. The initial impact of rabbit hemorrhagic disease on European rabbit populations in South Australia. J. Wildl. Dis. 34, 221–227.
Nagesha, H.S., McColl, K.A., Collins, B.J., Morrissy, C.J., Wang, L.F., Westbury, H.A., 2000. The presence of cross-reactive antibodies to rabbit haemorrhagic disease virus in Australian wild rabbits prior to the escape of virus from quarantine. Arch. Virol. 145, 749–757.
Neill, J.D., 1992. Nucleotide sequence of the capsid protein gene of two serotypes of San Miguel sea lion virus: identification of conserved and non-conserved amino acid sequences among calicivirus capsid proteins. Virus Res. 24, 211–222.
Ohlinger, V.F., Haas, B., Meyers, G., Weiland, F., Thiel, H.J., 1990. Identification and characterization of the virus causing rabbit haemorrhagic disease. J. Virol. 64, 3331–3336.
O'Keefe, J.S., Tempero, J.E., Motha, M.X.J., Hansen, M.F., Atkinsona, P.H., 1999. Serology of rabbit haemorrhagic disease virus in wild rabbits before and after relase of the virus in New Zealand. Vet. Microbiol. 66, 29–40.
Parkes, J.P., Norbury, G.L., Heyward, R.P., Sullivan, G., 2002. Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997-2001. Wildl. Res. 29, 543–555.
Parra, F., Prieto, M., 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. 64, 4013–4015.
Prasad, B.V., Rothnagel, R., Jiang, X., Estes, K.S., 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J. Virol. 68, 5117–5125.
Rasschaert, D., Huguet, S., Madelaine, M.-F., Vautherot, J.-F., 1995. Sequence and genomic organization of a rabbit hemorrhagic disease virus isolated from a wild rabbit. Virus Genes 9, 121–132.
Robinson, A.J., Kirkland, P.D., Forrester, R.I., Capucci, L., Cooke, B.D., Philbey, A.W., 2002. Serological evidence for the presence of a calicivirus in Australian wild rabbits, Oryctolagus cuniculus, before the introduction of rabbit haemorrhagic disease virus (RHDV): Its potential influence on the specificity of a competitive ELISA for RHDV. Wildl. Res. 29, 655–662.
Rodak, L., Smid, B., Valicek, L., Vesely, T., Stepanek, J., Hampl, J., Jurak, E., 1990. Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins. J. Gen. Virol. 71, 1075–1080.
Strive, T., Wright, J.D., Robinson, A.J., 2009. Identification and partial characterisation of a new lagovirus in Australian wild rabbits. Virology 384, 97–105.
Strive, T., Wright, J.D., Kovaliski, J., Botti, G., Capucci, L., 2010. The non-pathogenic Australian lagovirus RCV-A1 causes a prolonged infection and elicits partial cross-protection to rabbit haemorrhagic disease virus. Virology 398, 125–134.
Tan, M., Meller, J., Jiang, X., 2006. C-terminal arginine cluster is essential for receptor binding of norovirus capsid protein. J. Virol. 80, 7322–7331.
Trout, R.C., Chasey, D., Sharp, G., 1997. Seroepidemiology of rabbit haemorrhagic disease (RHD) in wild rabbits (Oryctolagus cuniculus) in the United Kingdom. J. Zool. 243, 846–853.
White, P.J., Norman, R.A., Trout, R.C., Gould, E.A., Hudson, P.J., 2001. The emergence of rabbit haemorrhagic disease virus: will a non-pathogenic strain protect the UK? Philos. Trans R. Soc. Lond. B 356, 1087–1095.