Chapter

Viral Disease in Lagomorphs: A Molecular Perspective

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Abstract

Our understanding of molecular biology of the viruses that infect lagomorphs is largely limited to the leporipoxvirus myxoma virus (MYXV) and the lagoviruses rabbit haemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV) that infect the European rabbit (*Oryctolagus cuniculus*) and the European brown hare (*Lepus europaeus*) respectively. Thanks to the great effort of historic surveillance studies and careful sample archiving, the molecular evolution of these viruses is being resolved. Although historically considered viruses that cause species specific diseases recent reports show that several lagomorphs may now face the threat of these maladies. The driving factors behind these changes has not been determined and the effect of these species jumps on lagomorph populations has yet to be seen. Lagomorphs are also affected by several other lesser studied viral diseases. In addition, recent metagenomic studies have led to the identification of novel lagomorph viruses the importance of these to lagomorph health remains to be fully determined. In this chapter we summarize molecular aspects of viruses that infect lagomorphs, paying particular attention to recent interspecies infections.

Keywords: myxoma virus, rabbit hemorrhagic disease virus, lagomorphs, molecular virology, rabbit, hare, species jump

1. Introduction

Two viral diseases affecting Leporidae are listed in the World Organization for Animal Health Terrestrial Manual for animals, myxomatosis and rabbit hemorrhagic disease. Both are of major significance to wild and domestic rabbits and hares causing important environmental harm with significant financial consequences. This is also reflected in the magnitude of scientific literature regarding these diseases and the viruses that cause them namely myxoma virus (MYXV) and rabbit hemorrhagic disease virus (RHDV). MYXV and RHDV belong to two virus families the *Poxviridae* and *Caliciviridae* respectively. These virus families include a number of other viruses that are of consequence to lagomorphs. In the first section of this chapter we consider the molecular biology of viruses from these two virus families. Lagomorphs are also subject to a broader range of viral infections that we shall outline in part II of the chapter.

2. Viruses that infect lagomorphs Part I

2.1 Leporipoxvirus (virus family Poxviridae)

2.1.1 A brief history; species jump and evolving virulence

There are currently two *leporipoxvirus* (virus family *Poxviridae*) of consequence for lagomorphs, myxoma virus (MYXV) and rabbit fibroma virus (RFV). Both cause tumorifications in their natural hosts *Silvilagus brasiliensis* (the Brazilian cottontail) and *Sylvilagus floridanus* (the eastern cottontail rabbit), respectively. However, they lead to considerably different outcomes following infection of the European rabbit (*Oryctolagus cuniculus*). RFV may cause myxomas in the European rabbit but animals mount an immune response and recover, MYXV however causes lethal myxomatosis.

Myxomatosis was first observed by Sanarelli [1] in European rabbits (*Oryctolagus cuniculus*) imported to Uruguay. Local rabbits (*Silvilagus brasiliensis*) (tapeti) were the source of the virus that caused this devastating disease. The disease is one of the best studied examples of a virus species jump or cross-species transmission. The emergence of myxomatosis in the European rabbit in the broader context is directly linked to human activity through deliberate releases of infected animals on three continents. The aim was to control populations of rabbits considered pests in Australia, France and Chile. (reviewed in [2–5]). Seventy years since its introduction the disease continues to persist. Through extensive monitoring programs and comprehensive virulence studies an evolutionary model of host-pathogen interactions was developed (reviewed in [5]).

Following the introduction of the disease in Australia attenuated strains of the virus soon arose [4] this was accompanied by the selection of more resistant rabbits. The comparison of rabbit genomes from before and after the introduction of MYXV has given considerable insight into the host resistance mechanism [6]. Immune related genes have been identified as being determinant in a complex interplay of numerous genes [6]. Based on survival times of laboratory rabbits inoculated with different virus isolates Fenner and colleagues defined five virulence grades 1–5 [4]. Virus isolates of virulence grades 1–2 have over 95% case fatality rates with survival times of less than 13 days for grade 1 or up to 16 days for grade 2 virus. Rabbits infected with grade 5 virus survive while grades 3 and 4 average survival time is 17–29 and 29–50 days respectively. Experimental studies in the decades following virus release showed grade 3 viruses to be most common in both Australia and Europe. Changes in virus virulence occurred rapidly, with less virulent strains detected after one year [4] and a comparison of results from Australia and Europe showing a common trend and highlighted the importance of insect vectors and weather conditions on selection of virus attenuation [4, 5, 7, 8].

2.1.2 Control and prevention

Myxoma virus (MYXV) is the virus of reference for the *Leporipoxvirus* genus. Poxvirus virions are large with a brick or ovoid shaped morphology (ViralZone). Replication occurs in the cytoplasm of infected cells. (reviewed in Fields Virology).

The MYXV genome is a linear double stranded DNA molecule of 161.8 kb encoding 171 open reading frames (ORFs). The ends of genome are covalently closed forming hairpin terminal loops. The genome of MYXV Lausanne was sequenced completely in 1999 by Cameron et al. [9] and contains inverted terminal repeats (ITRs) of 11.5 kb encoding 12 genes which are therefore diploid. The MYXV genome contains an abundance of genes with potential immunomodulatory

functions contained largely in the ITRs while genes the central core of the genome are required for virus replication, transcription and morphogenesis (**Figure 1**). Genes found in this central region are highly conserved among the poxviruses [12].

There is no treatment for the disease therefore only preventative measures are effective. As the disease is spread most commonly by biting insects (fleas, lice, ticks and mosquitos), insect control has fundamental importance, being the first line of defence on the farm setting. The introduction of new individuals on to a farm or wildlife area should be preceded by quarantine measures and animals showing clinical signs should be isolated. However, the only effective measure for virus control is currently vaccination.

Several vaccines exist against myxomatosis and these can be divided into two types; heterologous and homologous with both types being live attenuated vaccines. The heterologous vaccines use RFV (also denominated Shope fibroma virus). RFV can be used to provide protection against myxomatosis [13] as it is immunologically related to MYXV [14]. However, protection lasts a short time and the heterologous vaccines are considered less immunogenic. Heterologous vaccination is often used to vaccinate juveniles for the first time whilst subsequent vaccinations are administered using homologous vaccines. The homologous vaccines are based on laboratory MYXV strains that have been passaged in embryonated eggs or cell cultures until an attenuated phenotype is attained [15–17]. Several homologous vaccine strains are available in Europe (eg. SG33, MAV, Borgi, Leon162 among others). Protection with homologous vaccines offers longer lasting immunity than with the heterologous vaccines. Myxoma virus causes immunosuppression in rabbits [18] and one of the main drawbacks to the use of homologous vaccines may be associated with such immunosuppression [18, 19] that could exacerbate underlying subclinical infections.

The genome sequences of various vaccine strains have been studied. While there is no consensus on individual gene mutations that cause the attenuated phenotype in vaccines, several have large regions of the genome absent when compared to the parental strains (**Figure 1**) [11, 20, 21]. For example, the MYXV vaccine strain SG33

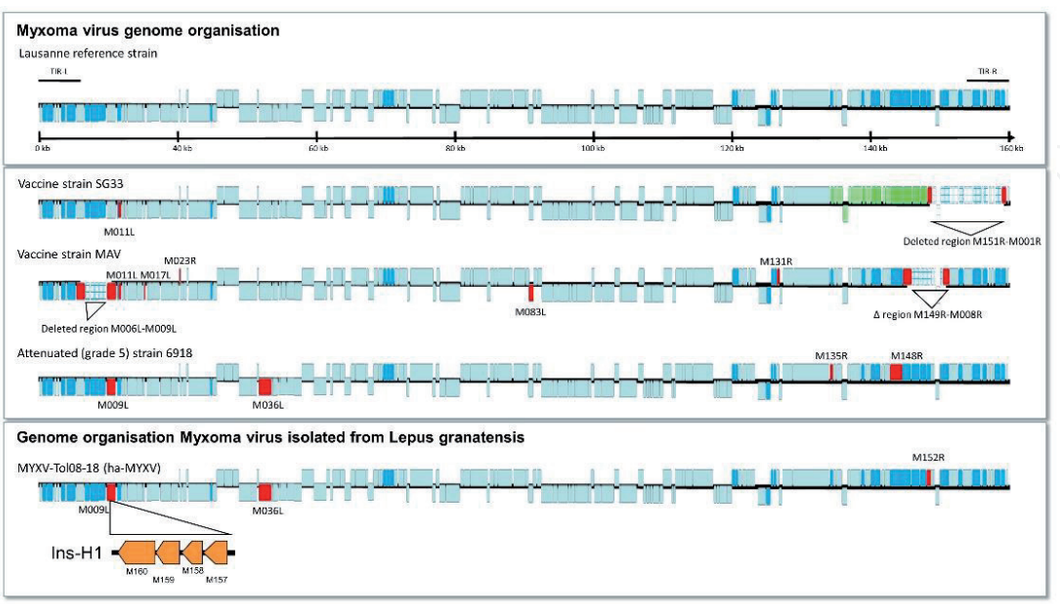

Figure 1.
Schematic representation of MXYV genome organization. Genes below the black line are designated L and those above the line are R. Immunomodulatory proteins of MYXV shown in darker shade of blue [9, 10]. Genes colored green indicate homology with California (MSW/ MSD) strains [11]. Genes showing major disruptions in attenuated strains shown in red and deleted regions indicated. Ins-H1 insertion in MYXV-Tol08-18 shown in orange.

has a genome 13.5 kbp shorter than Lausanne. The deleted region contained genes encoding for M150-M001 proteins (13 genes) including several genes expressing immunomodulatory proteins. The region affected contains a large part of the ITR and the right end of the central genomic region, while other mutations are also present in the genome notably in the open reading frames M011 and M077. SG33 was derived from a French field isolate and subsequent analysis of the genome revealed it to be a recombinant consisting of genomic sequences of the south American Lausanne and the Californian MSW/MSD strains [11]. The MAV vaccine strain (derived by serial passage of strain MSD isolated during an outbreak in California [22] has a 14.2 kbp deletion with respect to Lausanne, affecting both terminal regions flanking the ITRs (genes M006L-M009L and M148R-M008L/R). Interestingly, one European field strain has been sequenced that also contains a large deletion including part of the ITR, isolate Munich-1, this strain is described as virulent. Therefore, the precise mechanisms of attenuation of vaccine strains remain undefined however, the role of the large deletions that include genes involved in immunoregulation seems evident.

The publication of the MYXV genome coupled with advances in recombinant DNA technology and *in vitro* poxvirus manipulations paved the way for the design of recombinant attenuated strains and the development of potentially safer vaccines and may offer the possibility of reducing immunosuppressive effects. The deletion of virulence factors and the development of viruses with attenuated phenotypes [23, 24] has provided key information for the possible development of recombinant vaccines based on targeted attenuation.

Novel recombinant bivalent vaccines have been developed using MYXV as a vaccine vector that enable simultaneous vaccination against MYXV and other pathogens in domestic [25, 26] and potentially wild rabbits [27, 28]. The Spanish field strain 6918 showed great promise as a potential vaccine candidate in wild rabbits. A field trail of this vaccine showed that 50% of contact rabbits generated antibodies over a 32-day period. This isolate has also been used as a vector to express the capsid protein of rabbit haemorrhagic disease virus. The potential use of MYXV as a vaccine vector is not limited to use in rabbits. Indeed vectors have been tested in different species [29–31] including humans for use as an oncolytic virus vector (reviewed in [32]).

Outbreaks of myxomatosis still occur in farmed rabbits although efficient vaccines have been available since the 1980s. One major problem is that field strains are endemic in feral rabbit populations. There is therefore a constant source for virus introduction onto farms through uncontrolled insect vectors. Such outbreaks lead to doubts over the efficacy of current vaccines and the causes of these vaccine breaks have been investigated. Sequence data of field strains causing farm outbreaks show that the virus has not changed sufficiently to render vaccines outdated, indeed under laboratory conditions a traditional vaccine strain proved protective if the vaccinated rabbit generated antibodies [33]. However, the route of administration appeared to be a crucial factor. In this study a group of animals vaccinated subcutaneously failed to seroconvert and were susceptible to fatal infection, while animals vaccinated intradermically showed seroconversion and were protected. The key to controlling such phenomena on the farm setting is surveillance of antibody response following vaccination.

The two strains of the virus initially released in Europe and Australia have been completely sequenced. The reference strain (isolated in Brazil in 1949) known as Lausanne was released in Europe, while the Standard Laboratory Strain (SLS) also termed Moses strain was isolated in Brazil in 1910 and maintained by passage prior to its release in Australia (Moses 1911, reviewed in [5]). The fact that the date, locations, and viral sequence of released strains are known allows for the comparative

study of past and present MYXV sequences. Such precise data is unique and have allowed for continental scale virus evolution studies [21, 34, 35]. These studies have demonstrated that MYXV showed high mutation rates, frequent loss of ORFs due to nucleotide insertions or and deletions [21, 34, 35]. The evolution of complete genome sequences of MYXV strains over more than 70 years coupled with the data on virulence and virus phenotypes provided by disease monitoring programs [4] provide a powerful tool for the detailed analysis of the molecular mechanisms of virulence and attenuation. In addition, the study of recombinant knockout viruses, viruses with individual genes removed under laboratory conditions, and subsequent analysis of changes in virulence associated with gene knockouts allows the determination of these mechanisms with much greater precision (reviewed in [21]). Understanding the molecular mechanisms of attenuation will be crucial for the design of better control measures of MYXV.

2.1.3 Molecular mechanism of attenuation

Phenotypic studies of MYXV isolates demonstrated the emergence of attenuated field strains. Analysis of sequences of attenuated strains has revealed that virus attenuation is multifactorial. Such studies reveal there are varied pathways to the evolution of attenuation [2, 35]. Several genes have been shown to be involved but no single common mutation or group of mutations account for similar virus phenotypes. Genome wide analysis of MXYV strains isolated between 1952 and 1999 demonstrated the broad range of genes involved in MYXV evolution [34]. Strains of known virulence grades were included in such studies and although viruses shared virulence grades no common mutations between them were observed [34, 36] with similar results being obtained for Australian and European isolates [35, 36]. A subsequent study of isolates obtained between 2000 and 2021, (following the emergence of RHDV) showed a drastic change in virus evolution, echoing the effects that RHDV had on rabbit populations and highlighting the adaptability of MYXV to persist in the face of ever changing circumstances [37]. A more pronounced virus evolution was detected but once again the complexity behind attenuation/virulence were noted [37] as were the effects of RHDV and climatic conditions on the evolution of spread and transmission [37, 38].

The complexity behind the molecular explanation of attenuation is further exacerbated with the knowledge that individual or combination gene knockout studies have not precisely replicated attenuation in recombinant strains [39]. For example, of the Australian attenuated isolates, Uriarra contains an indel disrupting the M005L/R gene, while Meby codes for a truncated M153R gene. Both genes products play roles in immunomodulation [40, 41] and recombinant viruses with these genes deleted are less virulent (M005 highly attenuated, M153 moderately, [40, 42] respectively). However, reversion studies (replacing defective genes with corrected homologs in the genetic background of the parental attenuated strain) failed to show differences in the virus phenotype. The role of additional subtle mutations occurring in other parts of the virus genome although more difficult to decipher may hold the key to understanding attenuation completely. In the case of European isolates, attenuated strains have also been identified, e.g. Nottingham4-55/1 and the Spanish isolate 6918. Nottingham4-55/1 contains a truncated M150R gene. M150R (also termed myxoma nuclear factor (MNF)) is an immunoregulatory/host range protein [43] and one of the poxviral ankyrin-repeat (ANK-R) protein superfamily members. Recombinant virus with an MNF deletion is attenuated [44]. In addition to being attenuated, the Spanish isolate 6918 was poorly transmitted to contact rabbits demonstrating an additional phenotypic characteristic making it a possible safe vaccine candidate for use in wild rabbits [27, 28, 45]. Genome sequencing of isolate

6918 revealed mutations in four genes (M009L, M036L, M135R and M148R) leading to potential truncation of expressed proteins [46] (**Figure 1**). The M135R and M148R genes are likely candidates to affect virulence, while mutations in M009L and M036L genes have also been detected in virulent MYXV strains [47] however, the precise relation of these genes with attenuation has yet to be demonstrated. To date reversion studies using European strains have not been documented.

2.1.4 Diagnosis

In Europe the disease had a profound effect on rabbit populations including extinctions at local levels [48, 49] and much wider ranging ecological effects [49, 50] especially in the Mediterranean basin where the rabbit is considered a keystone species [51, 52]. Surviving rabbits show immunity [53] therefore disease maintenance requires sufficient susceptible naive juvenile rabbits and outbreaks therefore occur in temporal peaks which are also dependent on the availability of insect vectors which help to spread the virus (reviewed in [5]). In addition to the ecological effect, the rabbit industry (meat and fur) suffers substantial economic losses each year due to rabbit deaths and costs of control measures [54, 55].

Initial diagnosis may be based on the characteristic symptoms presented, however, disease caused by attenuated or respiratory strains maybe mistaken for bacterial infections. Laboratory diagnosis is therefore important and several methods are available for this task (reviewed in [56, 57]). Isolation of the virus in susceptible cell cultures (e.g. RK13 rabbit kidney cells) gives characteristic cytopathic effect but requires several days for confirmation and is therefore accompanied by serological or molecular techniques such as the detection of virus genome by PCR [20]. The most common samples for virus detection are tissue (eyelid, skin lesions, lung etc), swabs (conjunctival or genital) and sera for antibody detection. These techniques maybe complemented by electron microscopy, histology, agar gel immunodiffusion, fluorescent antibody test among others [57]. Detection of the virus genome requires extraction of viral DNA and subsequent PCR analysis and confirmation by sequencing may also be used. Targeted regions vary and include M153R [58] and M135R [59] or M005L/R gene within the TIRs (and therefore diploid) for qPCR [60] or conserved genes such as M022L [61] or M071L [20, 57] for conventional PCR analysis. Such analyses serve to confirm the presence of MYXV DNA, however, additional analysis of more regions are required to distinguish wildtype from vaccine strains [20] or in the absence of genome sequencing, to generate information regarding phylogeny [62, 63]. TIR regions in poxvirus genomes have been shown to be more variable [64], and RFLP analysis of long range PCR-amplified TIR regions from MYXV positive samples also allows for the molecular differentiation of strains [65].

Serological tests exist for the detection of anti-myxoma virus antibodies [19, 66, 67]. This analysis is of particular importance in farmed rabbits where vaccination is used.

2.1.5 MYXV jumps species to the Iberian hare (Lepus granatensis)

Myxoma virus is traditionally termed as being specific to the European rabbit. Soon after its release in Europe however, isolated cases were described in hare species (reviewed in [4]). More recently myxomatosis has been described in hares in the UK [68]. Experimental infections of hares (*Lepus europaeus*) with MYXV failed to result in disease [4] and natural cases seem to have been sporadic as no large-scale infections were described. These results do, however, demonstrate the susceptibility of hare species to MYXV infection, at least in certain circumstances. In spring 2018

the situation changed dramatically with reports of widespread infections in the Iberian hare (*Lepus granitensis*) occurring on the Iberian Peninsula [69–71]. Over 300 cases were reported and confirmed as positive for MYXV thus being the first large scale infection of hares with MYXV. The outbreak continued and spread over the Iberian Peninsula throughout 2018–2020 [72]. The impact this virus will have on the Iberian hare population has yet to be seen and must be continually monitored so that suitable control measures can be adopted. The virus has also been detected in wild rabbits (*Oryctolagus cuniculus algirus*) and commercial rabbits highlighting the need for control and monitoring [73].

Genomic analysis of the MYXV infecting the Iberian hare population showed the genome to contain an additional 2,8 kbp with regards to the reference strain Lausanne (**Figure 1**). How MYXV gained this genomic region is unknown, but homologous recombination has been demonstrated as a frequently occurring mechanism for poxviruses to gain selective advantage through the acquisition of genetic material from coinfecting viruses [74–76]. The genomic region present in the hare- infecting MYXV contained 4 full open reading frames which showed homology to MYXV genes M060R, M061R, M064R and M065R. The homologous region within the MYXV genome contains 6 genes, M060R-M065R, therefore homologs to M062 and M063 are missing from the hare specific genomic region. The nature of the ORFs included in the insertion led to two possible explanations: a duplication event of genes M060-M065 had occurred with the subsequent loss of ORFs M062 and M063 [70], or the capture by homologous recombination of this region from an as yet unidentified poxvirus [70, 71]. The exact cause of the species jump has yet to be explained, although the determination of the function of the gene products from within this region is sure to shed light on this phenomenon.

3. *Lagovirus* (family *Caliciviridae*)

There are two devastating diseases caused by lagoviruses that effect lagomorphs. Rabbit haemorrhagic disease (RHD) and European Brown Hare Syndrome (EBHS), as the names suggest, both were considered species specific. However, recent findings require that this view be revised. Both diseases have been endemic in Europe since the first descriptions in the 1980s (reviewed in [77, 78]). Due to the economic and ecological importance of the European rabbit, RHD has been the subject of most research. But the recent emergence of a lagovirus with broader host range, associated ecological concerns and advances in the molecular biological tools available for the study of these diseases, is changing this dynamic.

3.1 Rabbit haemorrhagic disease virus (RHDV)

3.1.1 RHDV GI.1

Rabbit haemorrhagic disease first came to light in China in 1984 in angora rabbits imported from Europe in what appeared to be the emergence of a novel highly virulent disease that rapidly killed thousands of domestic rabbits. The disease spread throughout China and Korea and reached Europe (Italy) in 1986 (reviewed in [77, 79]).

The first report of cases in Spain, the native home of the European rabbit was in 1988 [80] in domestic rabbits and the disease soon caused epizootic episodes in wild rabbits [81]. The reduction of the wild rabbit population was severe [82] and this had direct effects on specialist predator species such as the endangered Iberian lynx (*Lynx pardinus*) or Spanish Imperial Eagle (*A. adalberti*) [52, 83]. Such were

the effects of the disease on rabbit populations in Europe that tests were carried out for the use of the disease as an additional biocontrol measure against the rabbit pest in Australia, which was recovering following the initial success of myxomatosis in control efforts [84].

RHDV, the etiological agent of RHD, remained endemic in Europe for more than 30 years with a single serotype (RHDV GI.1) prevalent until 2010. Rabbits that survive infection generate a strong long lasting immunity with detectable anti-RHDV antibodies in sera. Young rabbits are not susceptible to the disease caused by RHDV GI.1, however, they may be infected and the mechanism of resistance is not fully understood. The economic effects of the disease in the rabbit farming sector are considerable. Inactivated vaccines (e.g. RHDV infected liver homogenates treated with β-propiolactone) were developed [85, 86] and proved successful in stemming mortalities in commercial rabbitries although due to the natural reservoir of RHDV in wild rabbits, control measures must be consistently maintained. With regards to control it is important to indicate that direct contact between rabbits [87] and fomites (contaminated food and bedding) play an important role in farm outbreaks. In natural infections, the faecal-oral route is considered the preferential mode of virus transmission [88] reviewed in [78]). In the wild, rabbit carcasses are also a major virus source with spread being facilitated by predators and carrion feeding insects [89, 90].

3.1.2 RHDV GI.2

In 2010 atypical outbreaks of RHD were detected. The virus responsible for these outbreaks was initially recognized in France [91] and termed a new variant of RHDV, where outbreaks affecting vaccinated rabbits caused concern. The variant was later detected in Spain, where isolates of the virus were used to show susceptibility of young rabbits, demonstrate major antigenic differences with regards to RHDV GI.1 and the presence of virus in the intestine [92]. Another considerable difference with the disease caused by this virus when compared to classic RHDV GI.1 is the level of mortality. RHDV GI.1 typically shows mortality rates of 80–90% in adult rabbits [93] with no mortality in kits, while the variant RHDV showed approximately 10% in adult rabbits and up to 50% in kits. The terms variant and RHDVb were first used to identify this virus [91, 92]. Subsequent publications used the terms RHDVb and RHDV2. In order to avoid confusion and bring order to the nomenclature system for the lagoviruses, Le Pendu et al., put forward a new scheme where by RHDVb/RHDV2 was termed *Lagovirus europaeus/GI.2*/ (GI.2), and RHDV was termed *Lagovirus europaeus/GI.1*/, (GI.1). The proposed nomenclature and classification system allows systematic definition using phylogeny and genetic distances to define isolates, includes pathogenic and non-pathogenic lagoviruses and allows for the incorporation of as yet unidentified virus sequences [94].

Given the novel characteristics of RHDV GI.2 it was unsurprising that the virus continued to spread. By 2013, the virus had been detected throughout France, Spain, Portugal and was present in Italy [95–97]. RHDV GI.2 and has since spread to pandemic proportions and has been detected on the continents of Europe, Africa, Asia, Australia and North America.

The lack of full cross protection induced by previous contact with RHDV GI.1 strains contributed to the rapid spread of RHDVGI.2 in Europe [98, 99], resulting in high mortality rates among wild populations soon after its emergence. Therefore, vaccines based on RHDVGI.2 containing inactivated liver extracts have been produced to aid in control of the disease in domestic rabbits.

The observation of partial protection between GI.1 and GI.2 was supported by data from RHDV GI.2 infections in Australia where mortality was detected in

RHDV domestic vaccinated animals [100] and contributed to its rapid spread in the wild [101]. Currently, it appears as though RHDV GI.2 has become the predominant RHDV on the Iberian Peninsula [102, 103], and also on mainland Australia replacing endemic strains of RHDV GI.1 [100]. While this may lead to environmental and economic benefits in Australia [104] the establishment of this virus in Europe poses an important problem for the conservation of the European rabbit, particularly in smaller populations [105, 106] and for the preservation of reliant predators [107].

3.2 Rabbit calicivirus (RCV) GI.3 and GI.4

The presence of anti-RHDV antibodies in rabbit populations is indicative of circulation of RHDV. However, in 1995, antibodies reactive with RHDV were detected in rabbits that showed no clinical signs of RHDV infection [108]. This finding was substantiated by the identification of a non-pathogenic calicivirus (termed rabbit calicivirus - RCV) [109]. RCVs were subsequently detected in Australia [110] and France [111]. RCV lagoviruses, are genetically related to RHDV although they demonstrate different cell tropism and are apathogenic. While RHDV target organs are lung, liver and spleen, RCV was found predominantly in the intestine. The presence of RCV has been argued as having a protective effect on rabbit populations facing RHDV outbreaks and may have been a determining factor in the speed of spread of RHDV in Europe and Australia [112], however the levels of protection vary and are likely dependent on differing levels of cross reactive and timing of infections [111, 113].

3.3 European brown hare syndrome virus (EBHSV) GII.1 and Hare calicivirus (HaCV) GII.2

European Brown Hare Syndrome Virus (EBHSV) GII.1 has been detected in many European countries and Argentina having emerged in Sweden in the early 1980s and Denmark in 1982 (reviewed in [114–121], although retrospective studies have demonstrated that the virus was present before this time [122, 123]. EBHSV causes disease in brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*) [114, 124], while the eastern cottontail (*Sylvilagus floridanus*) is also susceptible [125] but not the European rabbit (*Orytolagus cuniculus*) [126]. The disease symptoms are similar to those observed for RHDV, most notably the disease is acute and may show respiratory and nervous symptoms and epistaxis [127], the liver shows severe necrotizing hepatitis although differences between the diseases have also been reported [128]. Similar to RHDV GI.1, EBHSV does not infect young hares [121, 124].

The identification of hare caliciviruses (HaCV) GII.2 related to EBHSV have further shown the large diversity and complexity that exists for this genera of virus [129–132]. HaCV has been detected in duodenum or faeces of healthy hares in Italy, France, Austria, Germany and Australia (also weakly detected in liver) [129–133]. The virus is considered to be non-pathogenic based the health status of animals from which the samples were obtained and the target organ, however, virulence phenotypes have not been published. Expanding our knowledge on these viruses will undoubtedly help decipher their role in protecting populations of lagomorphs from pathogenic viruses and in the emergence of novel viruses through recombination events.

As lagoviruses EBHSV and HaCV have the same genome organisations (**Figure 4**) and virion morphologies as RHDV, however they form separate genetic groups following phylogenetic analysis and are antigenically different from RHDV [123, 134–137]. RHDV GI.2 has also been detected as highly virulent in different hare species [138] either as a result of spill-overs from closest rabbit populations or

from hare to hare transmission. These findings have highlighted the need for studies into virus cross species infections in lagomorphs [130, 132]. Recent detection of RHDV GI.2 and EBHSV GII.1 recombinant viruses [139] highlight the capacity for evolution of the lagoviruses and indicate the importance of continued vigilance in order to protect vulnerable lagomorph species.

3.4 Virion, genome organization

Lagoviruses are non-enveloped icosahedral single-stranded positive-sense RNA viruses [87, 140, 141]. As well as the morphological features of virions all lagoviruses share a common genome organisation, have conserved genomic features and express two ORFs as shown in **Figure 2**.

ORF 1 expresses a polyprotein that is processed to form non-structural mature peptides and VP60 the major structural capsid protein (also termed VP1 in the literature). ORF 2 encodes VP10 a minor structural protein, also termed VP2. Lagoviruses also share conserved polyprotein processing sites (**Figure 3**) and. Transcribe a VPg-linked subgenomic RNA from which VP60 and VP10 are expressed. The ORFs for VP60 and VP10 overlap and expression of VP10 occurs via a termination/reinitiation mechanism (**Figure 3**).

RHDV GI.1 is the lagovirus that has been most extensively characterised and is therefore the virus of reference for this genus. The virus genome is approximately 7.4 kb in length. Viral particles are small (35–40 nm diameter) and contain genomic (gRNA) and subgenomic RNA (sgRNA) which is collinear with the 3′ end of the genomic RNA [143–145]. Both RNAs are polyadenylated at the 3′ end. At their 5′ region they are covalently linked to the VPg (virus genome-linked) protein [146] (**Figure 2**) which may act as a substitute for cap during RHDV translation [147]. Genomic RNA contains two open reading frames (ORFs): ORF1 codes for a polyprotein of 257 kDa, which after a post-translational cleavage by a viral protease results in 7 non-structural proteins (p16, p23, p37 (NTPase), p29, p13 (VPg), p15 (protease),

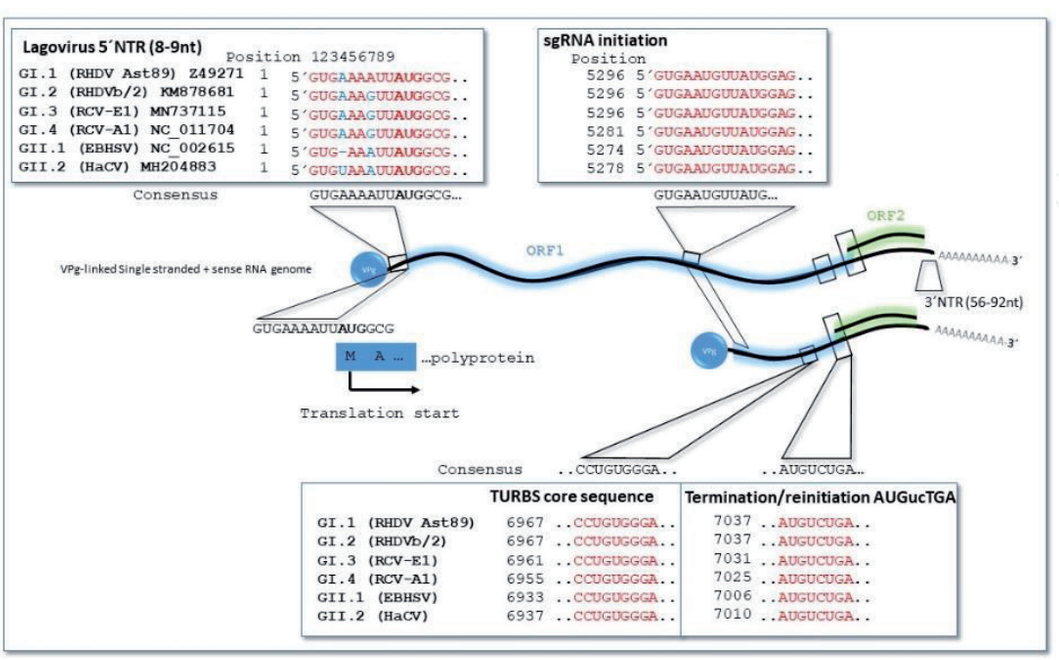

Figure 2.
Schematic representation of a lagovirus genome and the main genomic features shared amoung the lagoviruses. Fragments of sequence alignments from indicated isolates show: 5′ nontranslated region (NTR), polyprotein translation start site, subgenomic RNA initiation sites, termination upstream ribosomal binding site (TURBS) core sequence and termination/reinitiation site required for VP10 translation [142].

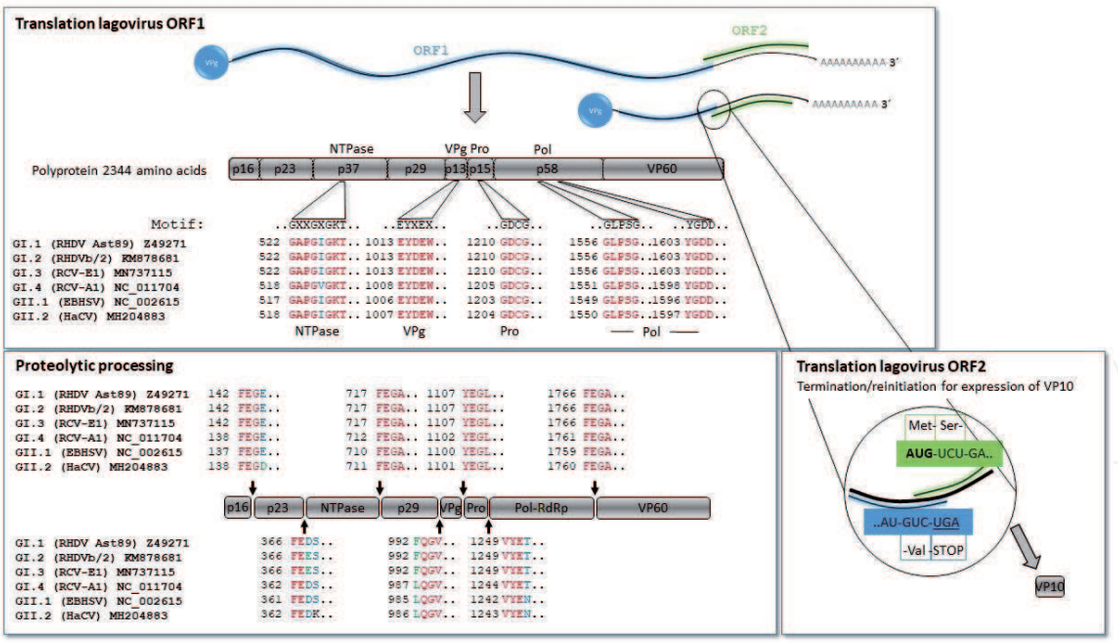

Figure 3.
Schematic representation of conserved motifs located in the ORF 1 encoded polyprotein and amino acid alignments showing conserved proteolytic processing sites among lagoviruses.

p58 (RNA-dependent RNA polymerase) (also termed non-structural proteins (NS) 1–7) and the major capsid protein (VP60) [144, 145, 148]. The RNA sequences encoding ORF1 and ORF2 overlap by 20 nucleotides. The reading frame of ORF2 is shifted with respect to ORF1 and codes for the minor capsid protein (VP10) which is 10–12 kDa. VP10 (also termed VP2 in some publications in accordance with norovirus nomenclature) is translated by a termination/reinitiation mechanism [142], dependent on a TURBS (termination upstream ribosome-binding site) element (core sequence GUGGGA) located within the VP60 coding sequence [149]. The role of VP10 protein has been linked with viral replication regulation and promotion of apoptosis for the liberation of virions from infected cells [145, 150]. The biological role of non-structural proteins has been studied by sequence analysis and functional studies, as well as being based on previous knowledge gathered from members of the *Picornaviridae* family [143, 145, 151]. Four of the nonstructural proteins have well defined functions, namely the RNA helicase (p37) [151], the virus genome linked protein VPg (p13), 3C-like protease (p15 or Pro) and the RNA-dependent RNA polymerase (RdRp) [146, 152, 153] (**Figure 3**). RdRp has been shown to catalyze VPg uridylation [146, 154]. The RHDV protease (Pro) [155] catalytic site has been mapped by site directed mutagenesis [155] and the target viral peptides have been identified [156]. The RdRp functions in replicating the viral RNA and VPg uridylation [154]. While the precursor (Pro-Pol) shows activity, the processed mature form shows increased capacity in polymerase function [154]. Expression of recombinant RHDV RdRp in transfected RK13 cells leads to Golgi membrane reorganization [157]. The crystal structure of this protein has been elucidated [158] allowing insights into the structural-functional mechanism involved in virus replication. The intracellular location of the RHDV non-structural proteins p16, p23 and p29 have been recently determined [157], however, their functions are still unknown.

3.5 VP60 major structural virion component

By far the most well characterized of the RHDV proteins is VP60. It is the major structural component of virions, responsible for receptor binding, is highly

immunogenic and the target of neutralizing antibodies. VP60 is expressed from subgenomic RNA in infected cells facilitating sufficient levels for virion assembly. High levels of sgRNA make it a good target for diagnostic RT-PCRs and its variability due to immune selection befit subsequent sequence analysis. Sequence data from partial and full-length fragments of VP60 encoding RNA have been the basis for comparative sequence analysis.

Although RHDV is not cultivatable *in vitro* the expression of VP60 and the formation of virus-like particles (VLPs) makes recombinant vaccine production an attractive alternative and ethically acceptable substitute for traditional inactivated vaccines which are prepared from infected liver homogenates.

VLPs have been used to characterize this protein structurally, determine its antigenicity and study receptor binding [159–164]. Additionally, purified wildtype virus particles have been used to determine an atomic model by cryo-electron microscopy [165].

RHDV GI.2 isolates are antigenically distinct from RHDV GI.1 and agglutinate human erythrocytes, to differing degrees, [92, 166–168]. Hemagglutination is dependent on the presence of ABH blood group antigens and these molecules have been proposed as receptor components of the binding pathway [169]. Indeed, the species specific nature of these molecules may explain susceptibility to different lagoviruses [170].

Virions are composed of 90 dimers of VP60 which form 32 cup-shaped depressions on the surface of virions (from which comes the name Calici- from the latin calix or cup) [165, 171]. Each monomer of VP60 consists of a shell (S) domain, which is buried inside the virion structure, and flanked by the N-terminal arm (NTA) and the so-called hinge which links the S domain to the protruding (P) domain that correspond to C-terminal region and is exposed on the virion surface [166, 172–174]. The hinge allows P and S domains a certain grade of flexibility on their tridimensional disposition [165]. The P domain contains determinants for virus-host receptor interactions and antigenic diversity [165, 166]. In addition, this region can be further subdivided into the subdomains P1 and P2, P2 contains the hypervariable region of the protein which allows for the selection of variants that can escape immune detection or antibody neutralization.

The marked antigenic differences observed between RHDV GI.1 and RHDV GI.2 [168], explain the lack of efficient protection against RHDV GI.2 afforded by RHDV GI.1 inactivated vaccines [91, 92], as well as, the capacity of RHDV GI.2 to overcome immunity derived from natural infections with RHDV GI.1 [101]. Since the emergence of RHDV GI.2 the development of diagnostic tools has been a very important issue and different techniques and methodologies have been improved for specific detection of RHDV GI.2 [168, 175–177].

3.6 Evolution: genotypes, antigenic variants

Although perceived as a novel virus (GI.1) in the initial 1984 outbreak, retrospective serological analysis [108] and sequence analysis [178–181] have cast doubt on this assumption. Molecular clock analysis suggest the virus emerged before it was officially detected [182]. These studies back the theory that RHDV or similar viruses existed in less virulent forms or went unnoticed and were circulating in the rabbit population long before the emergence of RHDV as a major concern [183]. Whether GI.1 and GI.2 arose from non-pathogenic viruses, through recombination events, or through species jumps remains to be determined [184–186].

Genetic diversity among pathogenic RHDV isolates has been the subject of intense study with the capsid protein (partial or complete) being the main target. Recent advances in sequencing techniques has allowed full genome analysis in

retrospective studies and demonstrate the importance of analysing complete genomes [187, 188].

Studies on the evolution of RHDV GI.1 in Europe showed low levels of sequence variation [183, 189] in the years following its emergence although it was possible to define distinct phylogenetic groups. In the 12 years following the emergence of RHDV GI.1 in France six genogroups were identified [190, 191] (now termed GI.1a-d). Genogroup 6 or RHDVa (GI.1a) is an antigenic variant first isolated in Italy [192]. Although antigenically distinct with a sequence similarity in the VP60 gene of 93%, GI.1 based vaccines provided protection against this highly pathogenic virus [191, 192].

The evolution of RHDV has followed a different trend in Spain compared to the rest of Europe. Only GI.1d strains (previously genogroup 1) have been detected in samples collected between 1988 and 2010. This may be due to a smaller number of samples being analysed, however, unlike in the rest of Europe, GI.1 strains were still being detected in the Iberian Peninsula in 2010. RHDVa was detected once in Spain in domestic rabbits [193] but does not appear to have been widespread and has not been detected in wild rabbits. In Australia the Czech strain V-351 (an RHDV GI.1) escaped from testing facilities in 1995. The sequence of virus circulating in Australia changed little in the first years following its escape [194], however, the study of RHDV sequences spanning the subsequent 16 years revealed its evolutionary tendency on this continent. Although subject of more than 3500 independent releases during this time, positive selection suggested the evolution of strains capable outcompeting freshly released strains and spreading in the presence of non-pathogenic rabbit calicivirus (RCV)-A1 (also GI.4a) [195]. In order to more effectively compete against the presence of RCV-A1, the antigenic variant RHDVa-K5 (GI.1a-K5) was released in 2014. Australian RHDV GI.1 strains gained virulence reflecting the selection of viruses [196] based on effective transmission, the immunological status of existing population, landscape and weather amongst others.

Phylogenetic analysis suggested that RHDV GI.2 is genetically distant from RHDV GI.1 and is more closely related to RCV apathogenic viruses [91, 92]. The origin of RHDV GI.2 is unclear and cannot be explained by genetic evolution from previously described lagoviruses or recombination of existing strains [197, 198]. Silverio and colleagues [197] using sequences analysis and mean substitution rates have estimated the presence of a common ancestor for GI.2 just a few years prior to its first detection [197]. Experimental infections have demonstrated that RHDV GI.2 has gained virulence showing higher mortality rates in both adults and kittens [98, 199], than the strains obtained in 2011 or 2012 soon after its emergence [98, 199]. This could indicate an evolutionary tendency of the virus, indeed, Capucci and colleagues (2017) [199] hypothesized that this could be similar to what occurred for RHDV GI.1 strains in Australia [196], RHDV GI.2 having evolved in their natural hosts and, since their emergence in 2010, selection pressure may have favoured strains with higher pathogenicity.

3.7 Recombination and lagoviruses

Recombination, along with mutation, is an important mechanism for the evolution of RNA viruses since it uses existing genetic diversity to create new genomic combinations. Novel RHDV virus genomes arising from natural recombination of existing strains have been detected in RHDV isolates [200–204]. Recent genomic analysis reveals that these events are more common than once envisaged [200]. and due to the high frequency of occurrence [195, 197, 200–203, 205] this might indicate an important role in their origin and emergence of pathogenicity. Indeed, Silverio and colleagues [197] indicated that the occurrence of recombination fits both

theories currently proposed for the emergence of pathogenicity in lagoviruses: (1) the evolution from a pre-existing non-pathogenic virus that acquired pathogenicity or (2) following a species jump [198]. Especially in the case of the highly frequent recombination events observed for the novel RHDVGI.2 and its ability to cross the species boundaries [99, 138, 206–208]. This also has been supported by Lopes and colleagues [187], who related the detection of a recombinant RHDVGI.2 in an Iberian hare with a species jump [187].

The number of combinations of genomes being observed is striking and reveals a complicated panorama. How these events shape the spread and pathogenicity of RHDV remains to be determined.

In recent years, recombination events in different regions of RHDV genomes have been identified. The existence of recombinant RHDV that contain structural genes from RHDV GI.1 and non-structural genes belonging to non-pathogenic lagovirus (GI.4) have been identified [202]. In some cases, the recombination event occurred inside the non-structural region [187]. Two types of RHDV GI.2 recombinant strains have been identified, both with their structural proteins VP60 and VP10 originating from GI.2, while their non-structural proteins originated from GI.1 or non-pathogenic strains (GI.4) [103, 187, 197].

Recombination has been reported in Iberian GI.2 genomes, with a breakpoint at the RdRp/VP60 boundary (**Figure 4**) within ORF1. This breakpoint was associated with several independent recombination events involving non-pathogenic strains, GI.1 and/or GI.2 resulting in different genomic combinations that persisted in the Portuguese wild rabbit populations [187, 197], and recombinant strains detected in Azores Islands and Australia [103, 202, 209]. The homology between the subgenomic RNA (encodes structural genes) and the 3′ end of the genomic RNA (encodes non-structural and structural genes) generates a hotspot of recombination at the junction between non-structural and structural genes [200, 202]. Silverio and colleagues [197] identified new RHDVGI.2 recombinants with a recombination breakpoint located near the p16-p23 (NS1/NS2) boundary (nucleotide positions 355–471), [197]. Also, they detected the occurrence of triple recombinants constituted by the NS1 non-structural protein similar to a nonpathogenic lagovirus, a

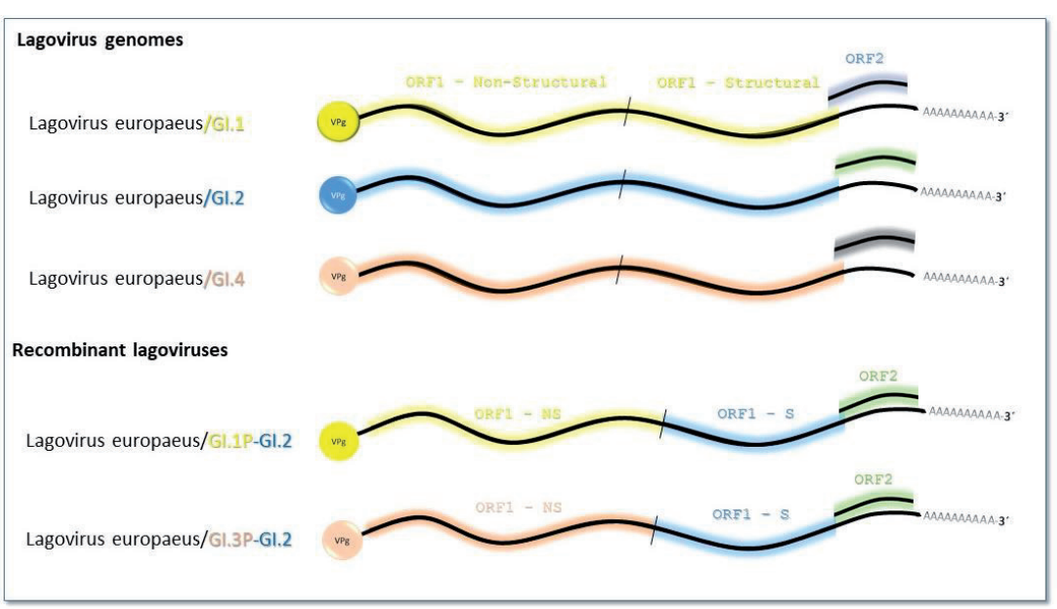

Figure 4.
Schematic diagram of recombinant lagovirus genomes. In the example shown recombinant Lagovirus europaeus/GI.1P-GI.2 indicates a recombination detected between a GI.1 and a G2 strain; P standing for polymerase.

GI.1b backbone for the remaining non-structural proteins and GI.2 as the donor for the structural protein. Mutations in NS1 sequences has been implicated as a factor in increased virulence of GI.1 isolates [196].

3.8 Cross species infections of lagoviruses in lagomorphs

Prior to the emergence of RHDV GI.2, lagoviruses were considered species specific, RHDV was specific to the European rabbit and EBHSV was specific to the European brown hare [126]. However, increasing numbers of RHDVGI.2 infections in different species of rabbits and hares have been reported casting doubt on this clear differentiation. Initially considered as transient spill-over events, more widespread infections of GI.2 in a number of hare species have raised concerns that this virus has an ampler species tropism. The first detection occurred in 7 Cape hares (*Lepus capensis* var. mediterraneus) with lesions consistent with those observed in RHD infections [99]. This study demonstrated for the first time that a *Lepus* species showed susceptibility to RHDV GI.2 and a clear difference with regards to susceptibility to RHDV GI.1 in this species given that RHDV GI.1 had previously been endemic in rabbits in the same geographical area for 20 years [99]. Subsequently RHDV GI.2 was determined as the cause of death of a captive Italian hare (*Lepus corsicanus*). In this species virulence was more limited, only infecting a single hare on the compound [206]. Velarde et al. [138] described the first infections of RHDV GI.2 in wild European brown hares (*Lepus europaeus*) detected in Italy (n=1) and Spain (n = 2). Serological data from wild hares presented in this study reinforced the finding that these were sporadic infections. However, widespread infections detected in France [210] showed that European brown hare infected with RHDV GI.2 were common in areas that hares and rabbits live sympatrically. Detection of GI.2 related mortalities in European brown hares in Australia [211], England [212] and Scotland [213] demonstrate that the problem is recurrent. An outbreak in Germany in captive mountain hares (*Lepus timidus*) adds to the list of susceptible species and demonstrated that juvenile mountain hares could succumb to the disease [214]. Reports of substantial outbreaks in the absence of local rabbit populations in Sweden demonstrate potential hare-to-hare transmission of RHDV GI.2 [215]. Wide spread deaths in different lagomorph species have also been reported from across the USA ([216] and news article therein) including black-tailed jackrabbit (*Lepus californicus*) and desert cottontail rabbits (*Sylvilagus audubonii*) [217]. Therefore, RHDV GI.2 exhibits a broader host range than classical RHDV (GI.1) by infecting not only different rabbit species but also different hare species (*Lepus capensis mediterraneus*, *Lepus corsicanus, Lepus europaeus*, and *Lepus timidus*).

Different species of hare infected by RHDV GI.2, showed very similar clinical signs typical of European brown hare syndrome (EBHS): hyperaemic trachea sometimes containing uncoagulated blood, hepatitis necrosis, splenomegaly and congestion of other organs and tissues [99, 138, 206, 215].

Retrospective studies have also blurred the line defining species susceptibility to RHDV and EBHSV. Lopes and colleagues [218], identified the presence of RHDV in archival samples from Iberian hares found dead in the 1990s in Portugal with signs of an EBHS-like disease [218]. These authors demonstrated that RHDV GI.1 strains in these two cases were phylogenetically closely related to those circulating at that time and in the same areas in rabbit populations. These results would support the theory of that virus dissemination and high infection pressure in the environment could favour spillover events of infection of European brown hares with RHDV GI.2 [99, 206, 210, 211].

Analysis of RHDV GI.2 positive hares sampled in 2013 [210] have shown the existence of co-infection by EBHSV GII.1 and RHDV GI.2. This is an important

issue in epidemiology and evolution, especially the potential emergence of recombinant EBHSV/RHDV GI.2 strains.

Species susceptibility to lagoviruses may be variable and this may reflect different species-specific host factors such as glycan expression for viral attachment [170]. With respect to this, several studies indicate that, as for noroviruses [219], specific binding between lagoviruses and glycans, particularly those of the HBGAs found in the upper respiratory tract and intestines of rabbits, is the first step of the viral infection [169, 220, 221]. Rabbits have different types of HBGAs and different virus strains show variable affinity to these molecules. Subtle changes in those attachment factors, e.g. through mutations, cause individual animals or even complete species can become more or less susceptible to the virus [169, 222, 223]. So, a possible explanation for overcoming species barriers could be the genetic variation of the capsid protein VP60 which alters the binding to histo-blood group antigens [223] that are considered as being important entry points for the virus [169, 221]. Other factors that could affect RHDV GI.2 infection in different species, such as concurrent subclinical infections, parasitic infestations, malnutrition or habitat detriment [138].

4. Viruses that infect lagomorphs Part II

A major concern regarding animal viruses are zoonotic infections. While viral zoonosis from rabbits or hares have not been commonly documented both, rabbits and hares are susceptible to infections that can infect humans including rabies, hepatitis E virus and herpes. Rabbit susceptibility to rabies was used to great benefit when Louis Pasteur endeavoured to create a rabies vaccine in the 1890s. Although susceptible to fatal rabies infection, rabbits are a spillover host. The most commonly documented source of infection in rabbits is from racoons, therefore the disease should be considered in areas where racoon rabies is endemic.

4.1 Hepatitis E virus

Hepatitis E virus has been detected in domestic and wild rabbits and the European brown hare in Europe and Asia, raising concerns as to whether lagomorphs maybe a reservoir for human infections [224, 225]. Although a recent study did not detect the presence of HEV in wild lagomorphs in Spain [226]. HEV belongs to the Hepeviridae virus family and has a non-segmented RNA genome comprising 3 ORFs. There are 4 genotypes, genotypes 1 and 2 cause human infections while genotpyes 3 and 4 affect wildlife species. Rabbit HEV is genotype 3. Liver or bile are the target organ for diagnosis using RT-PCR used to detect virus genome and specific ELISA to detect antibodies in sera. This pathogen should be considered when handling wild lagomorphs.

4.2 Herpes virus

Humans have been cited as the source of herpes virus infection in pet rabbits. With a fatal outcome the infections were the result of contact with a human with a cold sore lesion. Post-mortem analysis revealed HSV infection [227].

Naturally occurring herpes virus infections of lagomorphs have been detected in rabbit and hare species, as outlined in Chapter 1 of this book. Five putative species of Leporid herpesvirus have been described to date. Leporid herpesvirus types 1 and 3 have been isolated from *Sylvilagus floridanus* [228, 229] and Leporid herpesvirus types 2 and 4 have been isolated from *Oryctolagus cuniculus* [228–230]. Recently, during an outbreak of myxomatosis in the Iberian hare (*Lepus granatesis*)

leporid herpes virus 5 was reported [73, 231]. The most studied LeHV infection is caused by LeHV-3 which causes tumor-like lesions in various organs such as liver, spleen kidney and on lymph nodes [232]. The genome sequence of LeHV-4 has been published [233], this virus caused systemic illness that began with acute ocular infections in domestic rabbits [230]. LeHV-4 has been classified as a member of the *Alphavirus* virinae subfamily, genus *Simplexvirus*. While LeHV1–3 are related to members of the *Rhadinovirus* genus in the Gammaherpes virinae they have not been approved as species (ICTV 2019 release). It has been suggested that LeHV-5 is also a gammaherpes virus [73, 231].

4.3 Rabbit papillomavirus

There are two species of the papillomavirus that infect lagomorphs. The first, previously termed Rabbit Papillomavirus (Shope papilloma virus or cottontail rabbit papilloma virus), predominantly infects the cottontail rabbit (*Sylvilagus floridanus*) but may also infect *Oryctolagus cuniculus*. The virus is now termed Sylvilagus floridanus papilloma virus 1 and belongs to the *Kappapapillomavirus* genus (Van Doorslaer et al.) in the family *Papillomaviridae*. The virus replicates in skin tissue causing the growth of warts on the head and neck of rabbits which can become so large that they appear to be horns and can impede animal feeding if growth occurs close to the mouth. It was the first virus to be shown to cause cancer and up to 70% of warts will lead to cancerous growths. Vaccines have been developed for use in endemic regions and the virus/rabbit infection model has served as an excellent animal model for the study of antivirals and vaccines. Thanks to such studies much is known about the molecular biology of this virus. The *Kappapapillomavirus* genus also contains a second species of virus named *Oryctolagus cuniculus* papilloma virus 1. Infection of domestic rabbits with this virus causes self-limiting oral warts or papillomas that regress without treatment. Hares have also been reported to be susceptible to papillomatosis [234].

Several viruses have been implicated as contributing factors in rabbit enteritis complex (REC; also referred to as enterocolitis or enteritis complex). REC is a complex disease of the intestine in predominantly young rabbits although the precise causes are not known it is a multifactorial disease in which bacteria, virus, parasites and environmental factors are known to be important. The presence of several RNA viruses of notable concern has recently been analysed in this regard. Rotavirus, coronavirus, astrovirus and hepatitis E virus all cause enteric disease and may potentially be of concern [235].

4.4 Rabbit rotavirus

Rotaviruses are an important group of segmented-genome double-stranded RNA viruses of the family *Reoviridae* that cause gasterentroitis in mammals. Rabbit or Lapine rotavirus is a group A rotavirus and have been detected in several countries. Rabbit rotavirus infection has been implicated as a factor in "multifactorial enteropathy" [236]. In Spanish rabbitries, rabbit rotaviruses have been detected and found to often be associated with other pathogens such as *Eimeria* spp., *C. spiroforme, C. perfringens, E. coli* or combinations of these agents. The authors hypothesized that damage caused by rotavirus replication in the mucosa led to a predisposition for bacterial growth and infection [237]. Predominantly found in young farmed rabbits rotaviruses have also been described in Eastern cottontail rabbits (*S. floridanus*) and hares (Snowshoe and European hares). Molecular characterisation of lapine rotavirus strains requires RT-PCR analysis with regions of the VP4, VP6 and VP7 genes being targets. Several genotypes exist due to the capacity

for reassortment and genetic mutation that these segmented RNA viruses have. Detection of rabbit rotavirus in human infants have been reported [238, 239].

4.5 Rabbit coronavirus

Rabbit coronavirus was first described in 1961 following electron microscope detection of coronavirus particles and heart was described as the target organ [240]. Subsequently immunoelectron microscopy was used to detect Rabbit enteric coronavirus and virus was isolated [241]. Rabbit enteric CoV was detected in fecal matter during wet market surveillance in China. The characterisation of RbCoV provided the complete genome sequence (RbCoV HKU14-1 genbank accession number JN874559) has shown it to be a Betacoronavirus and reported cases detect RbCoV has also been implicated as a factor in REC. RT-PCR and RT-qPCR have been developed targeting the RdRp gene.

During the current SARS-CoV2 pandemic the potential for infection of animal hosts and the establishment of reservoirs has been of great concern. Molecular modelling studies suggest that the rabbit ACE2 molecule shares structural similarities with human ACE2 and could therefore act as a receptor for SARS-CoV2 virus entry leading to speculation that rabbit may be susceptible to infection [242]. Laboratory rabbits inoculated under experimental conditions with SARS-CoV2 were asymptomatic and low levels of infectious virus was recovered from nasal swabs up to 7 days post infection [243]. Such findings emphasize the need for strict biosafety control measures on domestic rabbit farms. At the time of writing no naturally occurring cases of SARS-CoV2 have been reported in rabbits.

4.6 Rabbit astrovirus

Astroviruses (family *Astroviridae*) are nonenveloped, with a single-stranded positive sense RNA genome. The discovery of astrovirus implicated in REC multifactorial disease highlighted the complexity of this disease and the need for continued surveillance [235]. Astrovirus was detected in healthy and symptomatic animals and the precise role of this virus in rabbit disease remains to be fully explored [235]. The qRT-PCR designed in this study targets the ORF1b (RNA-dependent RNA polymerase) region and provides the necessary tools for surveillance and phylogenetic analysis. Virochip coupled with metagenomic analysis allowed the identification and determination of the first full genome sequence of this agent associated with an outbreak of enterocolitis in domestic rabbits in the USA [244].

Metagenomic virome studies have shown the presence of astrovirus in rabbits from Australia and highlighted the potential for spread by insect vectors [245]. However, more analysis is required to determine the pathogenesis of this virus in rabbits.

Virome studies have also identified novel lagomorph bocaparvoviruses (genome sequence genbank accession number NC_028973) [246], picornavirus, caliciviruses amounst others [245]. The relevance of these viruses to the sanitary status of lagomorphs should be monitored. Such metagenomic studies offer novel insights into the viruses of these species and indicate the complexity of multifactorial conditions. The molecular tools that can be garnered will undoubtedly improve our understanding of the viral diseases of lagomorphs.

5. Concluding remarks

The detection of recent cross species transmissions of both MYXV and RHDV between lagomorph species, both sporadic and widespread and findings from the

analysis of historic samples are changing our view on the species susceptible to these diseases. Rabbit and hare species are genetically and immunologically similar and, in many regions, live sympatrically, key factors when considering virus species jumps. Soon after the release of MXYV in 1950 hares were known to be susceptible to this disease, however, no large-scale infections were documented until 2018. RHDV GI.1 emerged in 1984 in the European rabbit and this was the only lagomorph species apparently affected until the emergence of RHDV GI.2 in 2010. What has driven these changes to occur is a matter for study and the effect of these species jumps on lagomorph populations has yet to be seen. Thanks to the great effort of historic surveillance studies and careful sample archiving, the molecular evolution of these viruses is being discovered.

Metagenomic studies have also identified novel lagomorph viruses. Through such studies and continued surveillance therapeutics to lesser known lagomorph viruses and a better understanding of animal health will ensue. We may now be entering a new era in the study of the viruses that infect lagomorphs which will further our understanding on the complexity of virus-host relationship.

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Conflict of interest

"The authors declare no conflict of interest."

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