VIRAL HAEMORRHAGIC DISEASE: RHDV TYPE 2, TEN YEARS LATER

Capucci Lorenzo***, Cavadini P**atrizia**, Lavazza A**ntonio

OIE Reference Laboratory for RHD - Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Via Antonio Bianchi 7/9, 25124, Brescia, Italy
*Corresponding author: lorenzo.capucci@izsler.it

ABSTRACT

Until the early 1980s, it was totally unknown that lagomorphs were the hosts of several caliciviruses, which were included in the genus Lagovirus by ICTV in 2000. In those years, two new diseases appeared, with very similar clinical and pathological profiles and associated high mortality rates: rabbit hemorrhagic disease (RHD) in rabbits and European brown hare syndrome (EBHS) in European brown hares. It took a few years to ascertain that both diseases, actually acute and fatal hepatitis, were caused by two genetically related caliciviruses, but finally classified by ICTV into two distinct viral species on the basis of their molecular characterization and epidemiological data: RHDV in rabbit and EBHSV in brown hare.

RHD has had a devastating effect on rabbit farms, causing great economic damage, especially in China, where RHD was first noticed around 1982, and in Europe. RHD has also severely affected wild rabbit populations, whose drastic decline has caused serious ecological imbalances in territories, such as Spain, where rabbits are a central link in the wildlife food chain. Since the early 1990s, with the increased availability on the market of RHDV vaccines effective in protecting rabbits from RHD, the impact of the disease on rabbit farms has been significantly reduced. In the following years, also considering that RHDV is an endemic virus that cannot be eradicated, farmers learned how to manage the continuous use of RHDV vaccine in relation to the epidemiological situation, the type of breeding farm and the costs of vaccination prophylaxis. Although precarious, the management of the RHD risk for rabbit farmers reached an acceptable equilibrium, which was, however, completely upset starting from 2010, by the emergence of another lagovirus, also causing RHD.

The genome of the newly emerged virus shows limited differences with that of RHDV, but the phenotypic traits of the two viruses are distinctive in at least three main respects. 1) the antigenic profile of the virus (the "face" of the virus recognized by the antibodies) is largely different from that of RHDV; 2) newborn rabbits only a couple of weeks old die of RHD when infected with the new virus, while RHDV infections run asymptomatic until 7-8 weeks of age; 3) the new virus, which started in Europe, has spread over the years to several continents affecting wild and/or domestic rabbit populations. During this worldwide distribution, the new virus infected several species of lagomorphs and was shown to cause RHD in most of them. Considering these marked differences and the fact that the new virus is not a variant of RHDV, we proposed the name of RHDV type 2 (RHDV2). All these main distinctive traits that differentiate RHDV from RHDV2 have in practice the following consequences: 1) the antigenic difference between RHDV and RHDV2 (their 'faces') is so great that we need "new" specific vaccines to control RHDV2 (i.e. RHDV2 is a new serotype); 2) in the case of a RHDV2 infection of suckling rabbits, the presence of maternal antibodies to RHDV2 in its blood is the only way to prevent RHD. In contrast, newborns are naturally resistant to RHD if infected with RHDV and therefore, in terms of protection, the presence of maternal antibodies is useless; 3) when RHD outbreaks occur in territories where rabbits live in sympatry with populations of other lagomorphs, viral contamination in the environment reaches so high levels that facilitate the transmission of RHDV2 to other lagomorphs, including those with a lower susceptibility to infection than the rabbit.

Taken together, these phenotypic traits characteristic of RHDV2 are the reason for its rapid spread across the territory and the concomitant disappearance of RHDV. Probably the most striking example of the epidemiological consequences related to the peculiar features of RHDV2 is its rapid spread in USA and Mexico where is now practically endemic. There, despite repeated isolated outbreaks of RHD caused by RHDV from 2000 onwards in small rabbit farms, RHDV has never been able to become endemic.

Key words: rabbit hemorrhagic disease, virology, serotype, serology, diagnosis, prophylaxis

INTRODUCTION

Introduction to virology.

How many viruses on Earth? To this question Vincent Racaniello, one of the most eminent virologists, answers that "*The number rises to 100,939,140 viruses if we include the 1,740,330 known species of vertebrates, invertebrates, plants, lichens, mushrooms, and brown algae. This number does not include viruses of bacteria, archaea, and other single-celled organisms*." (Racaniello, 2013). Racaniello obtained this rough estimate starting from a recent work (Anthony et al., 2013) that, using a metagenomic approach, found 58 distinct viral sequences belonging to 7 virus family, in the Indian flying fox, *Pteropus giganteus*. Although these estimates are obviously very rough, surely each animal species harbours dozens of distinct viruses, belonging to several different families, with which they have "lived together" for thousands of years, through a continuous evolutionary process. Actually, the vast majority of these viruses are not cause of disease (i.e. they are not pathogenic) or, all the most, cause mild diseases.

Although there is a close relation between a virus and its host species (but some viruses "live" in more than one hosts) it must be kept in mind that both the virus and the host live in a specific and complex ecological niche. In addition, it should be also considered that viruses, especially those with few proteins that wrap around an RNA genome, have a huge capacity to evolve, and to change their phenotype, in very short time, in spite of the very low evolutionary possibilities of the host, practically, any in respect to the virus. Luckily for the host: a) viruses cannot live without a host, therefore usually the evolution selects for strains as much as possible contagious but also less pathogenic, to avoid the "suicide" of the virus itself; b) importantly, the host is protected from viral infections by an "almost perfect" immune system, therefore often the outcome of an infection is the results of the fight engaged between the host and the virus.

Moreover, it must be always considered that whenever the interaction between the single host and the virus is of course the necessary step for the virus replication and spread, the actual outcome of the infection and the related effect and damages caused, strictly depends on the degree of immunity at the level of host population. In other words, in a population largely vaccinated or that experienced since many years infections due to a specific virus, the virus spread is very limited, with a relatively low rate of infections in the specific host.

Finally, the first condition for a virus to infect the host is a close contact. Indeed, in the last decades the globalization level of the world increased a lot, with the consequence of consistent higher possibilities for a virus to come in contact with new potential hosts.

While we are writing this article, the Covid-19, a pandemic caused by SARS-CoV-2, a virus belonging to the Coronaviridae family, is in full swing, having caused more than 2 million of deaths around the world and forcing a large part of the human populations isolated in their houses, in order to prevent the "virus-host" contact and so slow down as much as possible the virus spreading. Today, more than a year after the beginning of the pandemic, the use of vaccines in developed countries is allowing us to return to an almost normal life, however, a prospect still far away for people living in poor countries. To note that this is not the first "spillover" of a coronavirus into humans. In November 2002, a viral respiratory disease first appeared in southern China and quickly spread to other countries, leading to over 8,000 confirmed cases and causing around 800 deaths. The etiologic agent was identified as SARS-CoV, a β-coronavirus, which disappeared, also thanks to the measures of containment of the epidemic, completely within a year. Again, ten years later another β-coronavirus, called MERS-CoV, emerged in Saudi Arabia as the causative agent of a SARS-like respiratory disease, counting actually over 2,000 confirmed cases and a mortality rate of∼35%. Genetic studies demonstrated that SARS-CoV and MERS-CoV originated in bats, jumping to human throughout a passage in intermediate vertebrate hosts respectively palm civet and camelids. Similar preliminary studies indicated that also Covid-19 (named SARS-CoV-2) is probably a zoonotic coronavirus jumped from bats, after a probable passage into an unknown intermediate host.

These three events occurred almost at ten year-period intervals, clearly shown that, for a complex of related causes not so easy to completely discover, bat coronavirus evolution have "open a door" towards a new host (humans), i.e. a way that allows them to repletely infect humans, thus trying "to earn/recruit" a new host species accounting for over 7 billion individuals, half of which amassed in

dozens of megacities with millions of citizens. The flare-up of the pandemic is evidence that the initial spillover of Covid-19 was successful, unlike the previous two, resulting in a definitive "species jump": today it is obvious to everyone that Covid-19 is a new virus of the human species, and it will remain so forever.

The "killer" viruses of rabbits: the myxoma virus (MYXV).

The European rabbit (*Oryctolagus cuniculus*) hosts several viruses belonging to different families (rotavirus, coronavirus, calicivirus, parvovirus, herpesvirus, papillomavirus, etc.) most of which are only mild pathogenic. However, two viruses, Myxoma virus (a poxvirus causing myxomatosis) and rabbit haemorrhagic disease virus (RHDV, a calicivirus causing RHD), are likely among the worst existing animal viruses, being highly contagious and causing mortality of over 90%.

Interestingly, the existence of the Myxoma virus, one of the first virus ever discovered, was firstly identified just because in 1889 Sanarelli observed the disease inside a little group of European rabbits, just imported in Brazil from Europe to breed and use in his laboratory. Actually, this is one of the many examples of the “globalization effect”, due to the translocation of one species, the rabbit, from its usual ecological niche (the Europe) into a totally new environment, where it came in contact with new microorganisms. Indeed, *Sylvilagus brasiliensis*, a lagomorph living in South America, is the natural host of Myxoma virus, in which it causes a no signs or a very mild disease. Therefore, the European rabbit encountered the myxoma virus, till then totally unknown, and the resulting spillover from the *Syilvilagus* was immediately successful inducing in European rabbits an overt and high pathogenic disease. Since then, the history of the relationship between the European rabbit and myxoma virus has been well known; in particular around 1950, myxoma virus was inappropriately exported by humans from South America both to France for controlling wild rabbit population, but from there it rapidly spread over all Europe killing tens of millions of the animals, and then to Australia to be used as biological agent in order to limit the damages caused to agriculture and the environment (Kerr et al., 2012).

The "killer" viruses of rabbits: rabbit haemorrhagic disease virus (RHDV)

It was back in fall 1986 when rabbit breeders in northern Italy would leave their healthy animals in the evening, only to find in the morning that sometimes largely more than half were dead, many with nosebleeds. Since then, all over Europe there has been a "massacre" of rabbits, both farmed and wild. In May 1989, the World Animal Health Organization (OIE) named the new disease viral hemorrhagic disease of rabbits (RHDV) and added it to the B List of the International Animal Health Code. As is often the case when faced with a new disease, it took the scientific community 2 years to agree that RHDV belonged to the Caliciviridae family, viruses with a positive strand RNA genome of about 7.5 kb, enclosed within a capsid consisting of 180 copies of a single protein (molecular weight 60 Kd) (Capucci et al., 1991; Ohlinger et al., 1990).

This conclusive statement regarding viral classification and etiology of RHD, beyond its intrinsic scientific value, paved the way to the development and use of a safe and efficacy vaccines. The availability of the vaccine was a key step in greatly reducing the negative impact of RHD on farmed rabbits: in fact, when and where the indirect prophylaxis is used properly, the risk of RHD to farms drops to a level of "almost negligible" However, the so huge populations acting as reservoir for RHDV, constituted by the wild rabbits and backyard farms, makes eradication of the virus impossible, and therefore, to keep low the risk of RHD, a continuous use of the vaccine over time is also essential. As a result, since about the mid-90s RHD, although not eradicated, has become a health problem at least manageable, subject of course to the negative impact on the economic balance of the farms related to the costs of vaccines used for prophylaxis.

Lagovirus family gets bigger: RHDV has good 'relatives'

In the early 1990s, with RHD already widespread throughout Europe, some laboratories developed serological ELISA methods. One of the first large sero-epidemiological survey was that reported by Rodak et al. (1990) in Czechoslovakia. They examined 43 rabbit farms and surprisingly, only 25% of the farms were completely negative, although they were never affected by RHD or had never been vaccinated. At that time, we also found that the rabbits reared in the experimental enclosure at our institution were ELISA positive for RHD antibodies, although never affected by RHD nor vaccinated.

Very interestingly, in both cases the seropositive animals were resistant to RHDV challenge, showing no signs of disease nor mortality. Few years later, we showed that the origin of this antibodies was due to the presence in the farm of a calicivirus strictly related with RHDV but not photogenic. We named this virus Rabbit Calicivirus (RCV) (Capucci et al., 1996). In subsequent years, two additional RCVs were identified: one (RCV-A1) in Australian wild rabbits (Strive et al., 2009) and a second in wild rabbits France in (RCV-Fra) (Marchandeau et al., 2005; Le Gall-Reculé et al., 2011b). Actually, the RCV-A1 is present also in European farms but, although genetically related to RCV-Ita, the outer shell of the virus, i.e. the external "face" of the virus recognized by the rabbit antibodies, is largely different from that of the European RCVs. Consequently, the vast majority of RCV-A1 infected rabbits die as a result of challenge with RHDV.
Indeed, genetic and serological data indicate that the viral members of the RCV group have been hosted by the rabbit population well before the emergence of RHDV, probably since centuries. Note that similar non-pathogenic Caliciviruses have also been found more recently in hares, both in Europe (Cavadini et al., 2015; Cavadini et al., 2020; Droillard et al., 2018) and Australia (Mahar et al., 2009).

RHDV2: A NEW PATHOGENIC LAGOVIRUS

France 2010: the emergence of RHDV2

In February 2011, people working in the rabbit sector were informed by a paper authored by Le Gall et al (2011a), that abnormal RHD cases have been identified in France in late 2010. Two main anomalies with regard to the "classical" RHD were alarming the researchers: a) farmed rabbits properly vaccinated with RHDV were not protected and were dying with a typical course and lesions as well as wild non-vaccinated rabbits, b) RHD deaths included bunnies, as young as 2-3 weeks old, which till that time were universally known as not susceptible to RHD. Thus, the Authors preliminary studied the virus agent of this "new" form of RHD and showed that it represented a "new genetic group". Two years later, the same authors published a further article together with us (Le Gall-Reculé et al., 2013) that included three further relevant findings: a) the antigenic surface (i.e the "print and face of the virus") was quite distinct from that of RHDV, b) the hemagglutination (HA) properties of the virus were similar to that of RHDV, c) the degree of pathogenicity of the virus was lower than that of RHDV, since average mortality in experimental studies was around 20%, but ranging from 0 to 50% in relation to rabbits and isolated strain used for challenge. In that paper, we concluded that the virus was not a simply "variant of RHDV", evolved from the previous RHDV, but a new real viral emergence. In addition, since 2011 several studies have reported the identification of this "new" RHDV in more lagomorph species, especially in hares, demonstrating a further difference in respect to RHDV which has been identified almost exclusively in rabbits demonstrating a high specie-specificity. For all these reasons, we decided to name the virus RHDV type 2 (RHDV2) (Le Gall-Reculé et al., 2013, OIE, 2021).

RHDV2 diffusion in the world: why RHDV2 has rapidly replaced RHDV?

In the span of a decade, RHDV2 has spread to all countries in the world where are present populations of lagomorphs (and not only rabbits, see below "Host range of lagoviruses"). To date, except for rare cases of RHDVa i.e. the most important and consistent variant of RHDV, identified on mid '90 (Capucci et al., 1998), all reported cases of RHD are caused by RHDV2. Below are detailed the main phenotypic characteristics that, being different from that of RHDV, allowed the "success" of RHDV2.

The antigenic profile of RHDV2 and the immune response towards RHDVs

The structure of calicivirus is made by 180 copies of one main protein of about 60Kd that folds to form a capsid with inside the RNA genome. The outer shell of the capsids is the "face" of the virus. Actually, it is formed by a limited number of amino acids codified by the VP60 gene.
In addition to hosting the site of binding to the cell receptor, the outer shell is recognized by the antibodies produced by the animal in response to the infection. A portion of these antibodies binds to the "face" of virus, stopping its replication and therefore preventing the disease: they are called "protective antibodies". Following genetic modification of the VP60 gene, some or several amino acids of the virus surface my change (i.e. the virus more or less changes its "face"). If the change is

limited, we have a variant classified as “subtype”. In practice, this means that if rabbits previously infected (or vaccinated) with the original virus are then infected with the “variant”, a high percentage of them do not develop RHD. This because a subset of the antibodies produced towards the original virus, still recognize and so neutralize the variant. On the contrary, if the number of modified amino acids exceeds a certain threshold, the variant virus changes its 'face' completely, and the antibodies induced by the original virus are no longer able to recognize and neutralize it. In this case the variant is classified as a “new serotype”. In practice, this means that rabbits previously infected or vaccinated with the original virus are “almost” fully susceptible to develop the typical signs and lesions of RHD with high percentage of mortality.

The genetic and antigenic data available on RHDV2 indicate that its “face” largely changed in comparison with that of RHDV (Capucci et al., 1995; Le Gall-Reculé et al., 2013). As consequence, a rabbit immune system “alerted” towards RHDV (by previous infection or vaccination) is not able to stop the RHDV2 infection, and in most cases to prevent clinical RHD. This happens because the humoral response (i.e presence of specific antibodies) is the immune system's main weapon of defense against lagovirus if compared to the adaptive cellular immunity and the innate immunity. In fact, it is well known that even very low level of specific antibodies (anti-RHDV or anti-RHDV2) circulating in the blood stream, could stop the virus replication and prevent RHD.

All pathogenic RHDVs replicate in the liver causing a lethal and acute hepatitis: after the initial infection, that presumably starts at the mucosal level of the gastro-intestinal apparatus, RHDVs reach through the blood stream the liver. There the amount of RHDV virions rapidly increase in few hours with two consequences: a serious damage to liver function but also a huge input to the adaptative immune system, which very quickly activates the mechanisms of production of anti-RHDVs IgM. In experimental trials, , after 36-60 hours post infection using the oral route, there is a huge level of RHDV in the blood and most of the rabbits die for RHD. However, in few rabbits RHDVs replicate and increase a “bit” more slowly (just by change? or due to an innate immune system more able to contrast RHDVs? likely as effect of an intra-species genetic variability), just the time necessary to the rabbits to produce a first peak of IgM (around 72 – 84 hours post infection). In about half of these rabbits the “fight” between RHDV and IgM binding them, is won by IgM: in few hours RHDVs disappear from the blood and in few days these rabbits, sometimes passing through a short state of agony, recover from RHD. This shows the paramount importance that antibodies have in contrasting the RHDVs infection and for saving rabbits from RHD.

In 2010 large part of the rabbit populations, both wild and farmed, had from moderate to high levels of RHDV antibodies (so-called “herd immunity”). In addition, in the areas where wild rabbit populations were present with good consistency (e.g. in some regions of France) they endemically hosted European RCV (Stephane Marchandeau, personal communication) and therefore had additional antibodies that protected them, at least partially, against RHDV. In other words, after about 25 years form its first occurrence in Europe, the rabbit populations had achieved a fairly good level of herd immunity to RHDV. However, this immunity had limited effect against RHDV2, just due the change of its “face”, and this was surely one of the main factors that allowed RHDV2 to rapidly diffuse all over the world, causing the second wave of a devastating pandemic of RHD.

RHDV2 cause RHD also in young rabbits

During the first pandemic of RHD due to RHDV young rabbits (rabbits less than 7-8 weeks old) had two defense mechanisms against RHD: antibodies anti-RHDV in the blood, eventually inherited from the mother and a natural resistance to RHD, with sub-clinical RHDV infection. There are several evidences that indicate this is due to the ability, which is lost when the animal grows, of the innate immune systems of young rabbits to prevent and/or compensate by a rapid liver cells regeneration, the RHDV replication in the liver. Importantly, if rabbits are infected with RHDV at a young age, they develop a specific immune response with good levels of antibodies in the blood. In practice, they are “naturally vaccinated” and are most likely protected for life by RHD. From several data, the first already reported by Le Gall-Reculé et al (2011a), RHDV2 is able to overcome the innate immune response, replicating at high level also in the liver of kittens, so causing RHD.

This distinctive feature of RHDV2 considerably increases the number of animals susceptible to RHD, thereby increasing the viral load in the environment and thus facilitating the spread of RHDV2. This has been clearly demonstrated in a recent study on the diffusion of RHDV2 in Australia (Taggart et

al., 2021). By using a serological approach these authors showed that RHDV2 outbreaks are usually beginning in coincidence with the commencement of annual breeding cycles. Indeed, a large proportion of adult breeding rabbits are serologically positive for RHDVs as a result of infections acquired in previous years. Consequently, it is the emergence of newborns, i.e. of non-immune animals, that gives the virus the opportunity to cause the disease and the high viral load on the territory that is essential for its spread. The Authors showed that this is the key point by which RHDV2 has a considerable advantage over RHDV. Indeed, whereas RHDV2 causes RHD, RHDV causes only a subclinical infection with very limited viral excretion in the field contamination (contaminated faeces excreted for 1-2 weeks), certainly not comparable in quantity to that caused by a carcass of a dead rabbit with a 'full' virus load.

What about maternal antibodies?

In rabbits, maternal antibodies are transmitted from mothers to offspring during the gestation and/or lactation with the aim to protect during their early life. Maternal IgG are transmitted directly from the mother to the offspring through placenta. In this way newborns are passively protected from RHDVs that, once replicated at mucosal level, fail to initiate a systemic infection. Differently, maternal Ig (mainly IgA) present in the milk passively protect the mucosa from infection during the period of lactation.

Available data indicate that anti RHDVs antibody titres are detectable in the blood up to 6-7 weeks of age, however depending strictly on the value of the titres of the mother (Baratelli et al., 2020). At the light of the different susceptibility of young rabbits to RHDV and RHDV2, the importance of the contribute of maternal antibodies to the protection from RHD consistently changes. Indeed, as young animals are resistant to RHD until around 7-8 weeks of age, the presence or absence of maternal RHDV antibodies has little, if any, advantage. Conversely, as young rabbits are susceptible to RHD if infected with RHDV2, the presence of maternal antibodies against RHDV2 is of paramount importance to protect them from RHD during the first weeks of life. As consequence, in farmed rabbits it should be of primary importance to keep the anti-RHDV2 antibody titre in the does as higher as possible over time, in order to extend the presence of IgG in the serum for several weeks. This is in recognition of the fact that even minimal levels of specific antibodies in the blood protect rabbits from RHD.

The host range of lagoviruses

In the late 1990s, when the 'story' of pathogenic lagoviruses began, many people assumed that RHD in the rabbit and European Brown Hare Syndrome (EBHS) in the European brown hare were diseases caused by the same virus. Those who thought so had more than one reason: a) the two diseases emerged more or less at the same time; b) the clinical and pathological findings were very similar; c) the two affected animal species belonged to the same family (Leporidae); d) the etiological agent identified was a calicivirus in both cases. However, early studies of the genetic and antigenic characterization of the two viral agents showed that they were distinct, even if antigenically correlated, caliciviruses (Capucci et al., 1991; Wirblich et al., 1994). It is important to note that in subsequent years and up to the present day, experimental replications of the two diseases in rabbits and hare, as well as epidemiological data collected from both virological diagnosis and serological investigations have shown that RHDV infects and causes RHD only in rabbits and EBHSV infects and causes EBHS mainly in brown hares (Lavazza et al., 1996) and to a limited extent could also cause disease in cottontails (*Sylvilagus floridanus*) (Lavazza et al., 2015). At the light of these results, in 2000 the International Committee on Taxonomy of Viruses (ICTV) created a new genus in the Calicivirus family designed Lagovirus that includes two viral species: RHDV and EBHSV (ICTV, 2019).

However, just few years after RHDV2 emergence, it became clear that, although the rabbit remains the main host species, also several species of hares i.e. *Lepus capensis* var. *mediterraneus* (Puggioni et al., 2013), *Lepus corsicanus* (Camarda et al., 2014), *Lepus europaeus* (Velarde et al., 2016), *Lepus timidus* (Neimanis et al., 2018) could be infected developing an EBHSV-like disease Moreover, epidemiological data, unequivocally indicated that RHDV2 cases mainly occur in hare populations living in sympathy with high-density rabbit populations, when they are affected by RHDV2 outbreaks.

To fully understand the relevance of this differential trait between RHDV and RHDV2, we must consider what is happening in North America (Javier Asin Ros and David L. Bergman, personal

communications). Till 2018 the cases of RHD in North America were very few, mostly of them presumably due to introduction of rabbits from countries where RHD is endemic. The first cases of RHDV2 in North America date back to 2016 in Quebec, Canada. Then, in 2018, RHDV2 occurred in Vancouver Province, British Columbia, Canada with numerous outbreaks and a rapid spread, also favored by the presence of large populations of feral European rabbits. Several other detections, between 2018 and 2019 were in Washington State and Ohio, USA, in 2018 and 2019; and New York, USA, in 2020. However, the most widespread outbreak commenced in 2020 in the southwestern USA and northern Mexico, with detections in multiple states of the USA to date. During this overwhelming spread into new territories, RHDV2 caused RHD in several leporid species, including Antelope rabbit (*Lepus alleni)*, Desert cottontail (*Sylvilagus audubonii*), Mountain cottontail (*Sylvilagus nuttallii*), Eastern cottontail (*Sylvilagus floridanus*). Thus, the total epidemiological data on RHD in North America indicate that, despite several introductions, RHDV failed to become endemic while RHDV2 became endemic after only few attempts.

Finally, the epidemiological and genetic data on RHDV2, particularly those collected in Europe till now, suggest that, from the point of view of the host specificity, three is only one RHDV2. In practice, the RHDV2 that is causing RHD in rabbit is the same virus, able to infect and cause disease in multiple lagomorph's species. In other words, there are not yet data available suggesting that a "variant" of RHDV2 is preferentially circulating and evolving in lagomorph species other than rabbits, or in some of them.

The origin and evolution of RHDV2

As recalled above, SARS-CoV-2 was the third spillover of a coronavirus from animals to humans, the one that, through a probable adaptation process, gave rise to the pandemic that caused millions of deaths. Coincidentally, three new caliciviruses (RHDV, EBHSV, RHDV2) also appeared in lagomorphs between the early 1980s and 2010, and all them caused major epidemics in almost every continent where lagomorph populations live. All three viruses, in addition to being genetically related, cause very similar diseases (RHD and EBHS), in terms of clinical signs, lesions and pathogenicity, i.e. an acute and fatal in 50-90% of cases hepatitis. Diseases so noticeable, severe and typical that their definition as 'new diseases' i.e. never seen before, is beyond doubt.

Therefore, the question "where" RHDV, EBHSV and RHDV2 come from is obvious. However, as in the case of human caliciviruses, despite the numerous genetic data that have been acquired even for the lagoviruses, we have only hypotheses to date. The most obvious and now widely accepted suggests that the pathogenic lagoviruses (PL) originated by genetic mutation from the non-pathogenic lagoviruses (NPL) (see above). In fact, we know that lagomorphs harbor NPLs of different genotypes, and that the reason for their non-pathogenicity is that they are essentially enteric viruses: NPLs replicate mainly in the duodenum but do not pass the mucosal barrier and thus their replication in the liver is very limited, if any. This is why infections with NPLs have a clinically inapparent course, even if they do not escape the vigilance of the host's adaptive immune system at mucosal level, which responds with significant levels of specific antibodies.

However, the assumption that non-pathogenic viruses that evolved and lived for centuries 'in peace' with their hosts, suddenly generate highly pathogenic 'relatives' that kill the host, is at odds with one of the assumptions of virology: that viruses evolve over time from a pathogenic to a non-pathogenic behavior.

The most logical explanation is that the phenotypic character of 'pathogenicity' gives PLs a high selective advantage over NPLs in infecting and spreading in host populations. In practice, this means that a dead RHD rabbit, storing hundreds of milligrams of RHDV in its body for weeks or months, is a much more significant source of infection than the faeces released by an RCV-infected rabbit.

In addition, this hypothesis has some basis in the evolution that has followed RHDV2 from its origin to the present day. Genetic analysis based on the rate of change of the genomes of RHDV2 strains isolated worldwide (molecular clock analysis) indicates that RHDV2 was born 3-4 years before its identification in 2010. This is the time that elapsed from the first 'spillover' - in this rather peculiar case, from an enteric virus to a liver virus inside the same animal species - to the manifestation of the new phenotype, which probably occurred through a consecutive series of adaptive genetic mutations.

In Italy, we first detected RHDV2 in two related farms in northern Italy: in one farm, mortality due to RHD was low (around 20%), in the other it was only the veterinarian's scrupulousness in sending us a

dead rabbit that allowed us to ascertain the presence of RHDV2. Subsequent experimental infections with RHDV2 strains identified in 2010 and 2011, carried out in collaboration with French colleagues at ANSES, allowed us to confirm an average mortality of around 20%, but with a variability between experiments of 0 to 50% (Le Gall-Reculé et al., 2013). These mortality rates were also confirmed by observation in rabbit farms in France affected by RHDV2 (Bernadette Le Normand, personal communication). A few years later, investigations in RHDV2 affected farms here in Italy indicated a marked increase in mortality. To confirm this observation, we performed an experimental infection with RHDV2 isolates from 2014 and 2015 and found a mortality rate between 80-90%, similar to that associated with RHDV infections (Capucci et al, 2017). Overall, these data would confirm that the highly pathogenic phenotype has been positively selected during the evolution of RHDV2. Australian colleagues also came to a similar conclusion when studying the spread of RHDV in wild rabbit populations (Elsworth et al 2014).

A final note on the ability of RHDV2 to genetically mutate. In addition to the classic mechanisms of single-point mutations (i.e. change of a single amino acid) or insertion/deletion of a few nucleotides (i.e. addition or subtraction of 1-2 amino acids), the rapid and widespread dissemination of RHDV2 has made it possible to realize the importance of genome recombination within the lagovirus genus. Around 2015, some Portuguese colleagues discovered RHDV2 strains whose genome arose from recombination between RHDV2 and a distinct lagovirus (RHDV or RCVs). Interestingly, the point of recombination within the genome was always the same: right between the non-structural protein and the capsid protein. This mechanism certainly contributes to a great deal of genetic variability in RHDV2.

These data also support the high prevalence of lagoviruses in lagomorphs. Indeed, it should be remembered that one of the conditions necessary for recombination between two viruses is that they both infect the same cell at the same time and replicate within it. This means that, while RHDV2 was obviously the 'main donor' genome considering its high prevalence in the lagomorph population from 2011 onwards, the second lagovirus genome donor should also be present in the population with sufficient prevalence to allow the two genomes to meet within the same cell.

The diagnosis of RHDV2

With exception for those cases characterized by high mortality rates, the certainty of the diagnosis of RHD requires laboratory testing. Recourse to laboratory testing is mandatory if the etiological agent of RHD is to be established, whether RHDV or RHDV2. This is even though RHDV2 is nowadays the predominant virus, with rare cases of RHDVa. Virological diagnosis is easy to carry out, using the various specific methods available (RT-PCR, ELISA or immunohistochemistry) and considering that in acute RHD the liver contains high quantities of virus (OIE, 2021).

The serological diagnosis of RHD, on the other hand, is more complicated, particularly when accompanied by the question of which virus induced the antibodies, RHDV or RHDV2? Factors complicating the diagnosis are more than one: a) the partial antigenic correlation between RHDV and RHDV2; b) a certain variability in antibody response between individual rabbits; c) animals may be vaccinated simultaneously with RHDV and RHDV2 and/or then infected with RHDV2.

At the RHD OIE reference laboratory, we have developed a competition ELISA (cELISA) specific for RHDV2, in addition to the one previously developed for RHDV. cELISA has the highest specificity among the ELISA methods, that means the best method for detecting mainly the sub-set of antibodies that specifically recognize the outer shell of the virus (its “face”). Actually, by using in association these two cELISA it is possible in several cases to infer the origin of the antibodies, i.e. if they were induced by RHDV or RHDV2. These cELISAs, together with other ELISAs methods able to detect the specific IgM and IgA response, have been widely used both for epidemiological investigation of wild rabbit populations, but alsoi n farmed rabbits either after cases of RHD, especially for declaring the extinction of one outbreak or to determine the vaccination efficacy,

Prevention and control of RHD due to RHDV2

As written above, humoral immunity (i.e. specific antibodies) is the animal's main defensive system against RHD. Even low levels of antibodies are sufficient to prevent the disease but under the condition that they are highly specific and homologous for the infecting virus. Considering the consistent antigenic difference between RHDV and RHDV2, such that they are classified as two

serotypes, and the observations collected from the field in case of RHDV2 outbreaks, since the beginnings of the epidemy, it become evident the need to have two specific vaccines available: one for RHDV (already available since many years) and one for RHDV2. Actually, it took some years before RHDV2 vaccines were fully available on the market and this represented a major problem for rabbit breeders. Only on some occasions and in some countries, such as Italy, this lack of registered products has been overcome by the possibility to produce auto-vaccines to be used in a single farm after an outbreak.

The first RHDV2 vaccines were produced employing the same protocol set up for RHDV vaccines. As RHDV, also RHDV2 does not grow *in vitro* systems and so the production of the vaccine is based on the use of the livers of experimentally infected rabbits (or, in case of autovaccines, the livers of rabbits which died in the outbreak). Once inactivated, the viral matrix is mixed with adjuvants, and vials containing a variable number of doses are commercialized. In Italy, and, to our knowledge, also in France, Spain and most European countries, there are two main vaccines based on the use of inactivated virions present on the market: Filavac VHD K C+V produced by Filavie - France and Eravac produced by Hipra – Spain. Filavac is a bivalent vaccine containing RHDV and RHDV2 (a strain collected in France in 2012) that uses aluminum hydroxide as adjuvant. Eravac is an RHDV2 vaccine (a strain collected in Spain in 2013) that uses an oil adjuvant produced by Hipra.

More recently, a trivalent recombinant-type vaccine, Nobivac Myxo RHD Plus, marketed by MSD, became available on the market. The vaccine is based on the use of two attenuated Myxomatosis virus strains, different from each other just because one has inserted the RHDV VP60 capsid protein gene into its genome, whereas the second has inserted the RHDV2 VP60 gene. This is a live vaccine and the active replication of the Myxomatosis virus is a necessary condition for the immune system to be stimulated to produce antibodies against RHDV and RHDV2. According to the manufacturers' information, the protection of the animal is guaranteed for at least one year.

Considering the overall characteristics of RHDV2 and the long experience in the use of indirect prophylaxis for RHDV, which aspects would need to be revised?

As written above, differently from RHDV, RHDV2 causes disease even in few-weeks-old rabbits. Considering that vaccination is only possible in rabbits from 30-days-old onwards, and full protection requires at least seven days post vaccination, there is a window of about five weeks in which the young/fattening rabbits are at risk of RHD due to RHDV2. However, post-weaned rabbits could be protected in this period by maternal IgG, if, of course, the does are themselves properly vaccinated. The duration of maternal IgG in the blood of newborns is directly proportional to the maternal titre and can range from 2 to 6-7 weeks. However, the presence of IgG in the blood has also a negative side effect. In fact, it is known that in vaccinated young rabbits when anti-virus IgG are still present in the blood, also in relation to their quantity, the effect of the vaccine could be reduced, if not abolished. In traditional “organ” inactivated vaccines this effect has to be considered for each single virus. In the Nobivac Myxo RHD Plus, the effect has to be considered for both myxoma virus and, in addition, individually for RHDV and RHDV2. This because the presence of antibodies against Myxoma virus, before vaccination, is indicative of a previously vaccinated or Myxoma virus infected rabbit with an immune system already alerted toward the virus. This could result in reduced, or no replication, of the live vaccinal virus and also failure to produce the immunogens RHDV and RHDV2. As consequence, in order to reduce the negative side effect of maternal antibodies (so-called interference effect), it would be better to vaccinate young rabbits starting from 45-50 days of age. Alternatively, in relation also to the type of vaccine used for the does, a serological survey inside the farm could help to understand the level of antibodies respectively in the mothers and in the young and to decide when it is the right moment to vaccinate (i.e. when young are seronegative).

However, the normal practice in industrial rabbit farming is not to vaccinate growing rabbit given their short life cycle (approximately 70-77 days), when the situation on the farm is normal, i.e. good biosecurity measures are applied and there are no outbreaks of the disease in the area. Indeed, since immunity starts after about 7–10 days, vaccination could also be considered a quite effective post-exposure treatment, and it may be included in the emergency strategies applied when RHD occurs in a farm. Following an outbreak of RHD, and especially in the case of RHDV2, which could induce disease also in young animals, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, it is strongly recommended to vaccinate meat animals at the age of 30–45 days, because the incidence of re-

infection is very high. Only after several (>3) production cycles it is advisable to stop vaccination of meat animals. To verify the persistence of infective RHD inside the unit, a variable number of rabbits, starting with a small sentinel group, should not be vaccinated and then serologically checked.

CONCLUSIONS

RHDV is one of the deadliest existing pests considering its very high virulence, contagiousness, and diffusivity. The short history, about 40 years of Lagovirus is studded with evolutionary events, the last of which is the appearance of RHDV2, which is not a simple genetic variant of the previous 'classic' RHDV but in fact a new emerging virus.

Beyond the origin and evolution of the aetiological agents of RHD, this disease is of paradigmatic value in the study of pandemics due to its high pathogenicity, worldwide distribution and ability to infect various species of domestic and wild lagomorphs. Among these species, European rabbit is of paramount importance, since it is not only a wild animal, both in its natural habitat and in an invasive form, as in Australia, but also a companion animal and one used in laboratories, and finally a species of zootechnical interest and an important source of animal protein in developing countries.

In this context, the attention that the scientific world has dedicated to RHD and its evolution is fully justified; the results and knowledge that field and experimental research have provided allow precise identification and characterisation of the aetiological agent, but also better prevention and management of outbreaks.

Finally, the emergence of three distinct viral entity (EBHSV, RHDV and RHDV2) within a few decades cannot be considered as single random events. Although largely unknown, there have been a number of interrelated biological events that have contributed to the repeated 'emergence' of pathogenic lagoviruses that may not be over. For this reason, it is necessary to maintain a high level of surveillance and control on lagomorphs, based on close collaboration between public control and research institutions, private operators and international institutions such as the OIE.

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