VIRAL INFECTION OF RABBITS

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ABSTRACT

The three most important viruses of rabbits include: Myxoma virus (MV), the poxvirus that causes Myxomatosis, the calicivirus (*genus* Lagovirus) of Rabbit Haemorrhagic Disease (RHDV), and Lapine Rotavirus (LRV), which is an enteric agent. There are some other viral agents in rabbits (parvovirus, coronavirus, adenovirus, calicivirus (*genus* Vesivirus), enterovirus-like, reovirus, herpesvirus and coronavirus) but both their occurrence and their pathological value are negligible.

Myxomatosis was firstly introduced in Europe more than 50 years ago and still represents a current and real problem. Recent studies have been focused to determine the role and function of the over 100 genes of MV encoding structural and essential proteins; in particular the immunomodulatory MV (im-MV) proteins (virokines and viroreceptors, immune modulators and anti-apoptotic factor), involved in contrasting the host immune system response towards the MV infection. One of the main tools to control myxomatosis in endemic areas is use of the live attenuated vaccines that are able to induce traceable humoral immunity for a variable time even if the protection of rabbits from the infection is not fully guarantee. Therefore, thanks also to the knowledge on the im-MV proteins, a new family of biotechnology deleted vaccines will soon be produced and made available in a few years. These should be more safe and able to induce a wider immunity and permit to apply the DIVA strategy i.e. to use serology to ascertain if the anti-MV antibodies detected in a rabbits originate by an infection or a vaccination.

RHDV is a non-cultivable calicivirus that infects rabbits and causes an acute fatal hepatitis, firstly described in China in 1984. The first consistent antigenic variant called RHDVa, was identified in Italy and Germany in 1997. Nowadays it is present in most parts of Italy and its field prevalence has reached value over 50%. In Europe it has been reported between 1997 and 2004 in Germany, France, Malta and Hungary. Outside Europe, it was reported in Reunion Islands, USA and South America and, taking account of the RHDV genetic sequences deposited at the NCBI databank, its presence in China is also evident from 1985. More recently some other RHDV isolates presenting peculiar characteristics were identified. Based on their reactivity with MAbs these strains could be considered as further and separate steps of variation of the RHDVa, possibly classified as sub-variants.

The detection of seropositivity for RHDV in the sera of healthy farmed and laboratory rabbits taken between 1975 and 1985, which is approximately ten years before the occurrence of the disease in Europe, has suggested the hypothesis of the existence of one or more "non-pathogenic" viral strains antigenically related to pathogenic RHDV. Rabbit Calicivirus (RCV), the first of these non-pathogenic RHDV-like viruses identified in healthy rabbits, was detected in Italy in 1996. It is avirulent, replicates in the intestine at a low titre and presents a 92% genomic identity with RHDV. The diffusion of RCV in different areas of Italy has been evaluated in five consecutive serological surveys during the period 1999-2008, throughout the determination at slaughtering of anti-RHD antibodies in non-vaccinated meat rabbits from RHDV-free commercial rabbitries. The results clearly show that antibodies reactive with RHDV are present in several rabbit populations: almost 30% of controlled farms and over 80% of animals. The definitive proofs that an active infection had occurred came from the detection of IgA and IgM as well as the identification of viral strains by using PCR on faeces. In addition, the existence of other non-pathogenic caliciviruses in wild rabbits was suggested by the serological surveys of rabbit populations in European countries (UK and France), Australia and New Zealand. Either the identification of RNA particles related to RHDV in rabbit sera collected since 1955 in Britain and the

very recent isolation and identification of one of these viruses in Australia finally confirmed such hypothesis.
Enteric diseases have an important role in the rabbit industry since they produce severe economic losses due to mortality, growth depression and worsening of conversion index. Among the different pathogens that could be found in rabbits suffering from enteropathy, viruses seem to have an important but not definitive role. Viruses and among others Lapine Rotavirus (LRV) particularly, should not be able to induce primary episodes of high gravity but, acting as mild pathogens, they have the capacity of became endemic. The role and importance of viruses as primary aetiological agent of rabbit enteritis are here discussed, by both reviewing the available literature and presenting the results of studies of prevalence of the viruses identified in rabbits with enteritis. That is to recognise the main features and pathogenic abilities of different viral agents and to try to attribute them an etiological role in enteric syndromes, relating their presence with pathologic lesions.

Key words: Virus, Viral enteritis, Myxomatosis, Rabbit haemorrhagic disease, Epidemiology.

INTRODUCTION

The three most important viruses of rabbits include: Myxoma virus (MV), the poxvirus that causes Myxomatosis, the calicivirus (*genus* Lagovirus) of Rabbit Haemorrhagic Disease (RHDV), and Lapine Rotavirus (LRV), which is an enteric agent. In particular, MV and RHDV can cause severe losses and a huge economic impact due to high level of morbidity and mortality, and their occurrence in most countries is followed by the application of strict measures of health policy. The impact of LRV is lower but indeed it should be considered an important aetiological agent of the so-called "enteritis complex". These viral infections can be efficiently controlled and limited by a correct management plan through the use of hygienic measures of direct prophylaxis together with the application of specific vaccination programs.

There are other viral agents in rabbits, but both their occurrence and their pathological value are negligible. Most of them have been detected in rabbits with enteritis, *i.e.* parvovirus, coronavirus, adenovirus, calicivirus (*genus* Vesivirus*)*, enterovirus-like, reovirus, and are generally not considered as primary agents of disease. Herpesvirus and coronavirus (the agent of pleural effusion disease) can cause a systemic disease but they have been very rarely reported.

MYXOMATOSIS: STILL ONE OF THE MAJOR THREATS FOR RABBIT BREEDING

From a virological point of view, Rabbit Haemorrhagic Disease virus (RHDV) and Myxoma Virus (MV) are the main health and economical problems for rabbit farmers because both virus infections cause rapid, systemic and lethal diseases with a mortality rate often over 80%. Differently, while MV, illegally introduced into Europe more than 50 years ago from South America (Fenner, 1994; Fenner and Fantini, 1999), still represent a current and real problem, RHDV became a solved problem with the introduction of a reliable and efficient vaccine after its sudden and dramatic appearance. The main reason of this major difference is because RHDV and MV belong to two very distant virus families, characterized by peculiar strategies used to survive in the host over time.

MV belongs to the *Poxviridae* family, genus Leporipoxvirus with a very large linear double stranded DNA encoding 171 unique genes (twenty times more than RHDV!). The entire genomes of the South American strain, Lausanne (Cameron *et al*., 1999) and the North American strain MSW (Labudovic *et al*., 2004) have been sequenced. While the central part of the genome includes approximately 100 gene encoding structural and essential proteins, the extreme parts of the genome include many immunomodulatory genes involved in contrasting the host immune system response towards MV infection. Actually, successful MV replication and the consequent degree of disease induction are related to its ability to avoid recognition and clearance by the innate host and acquired immune system of the infected rabbits (Kerr and McFadden, 2002; Jeklova *et al.*, 2007; Stanford *et al*., 2007).

Immunomodulatory MV proteins (im-MV proteins) are included in three main categories in relation to the target specific pathways: 1) virokines and viroreceptors, 2) immune modulators and 3) anti-apoptotic factor (Stanford *et al*., 2007). Most of the im-MV proteins interfere in specific host pathways "miming" one of the host proteins involved in the transmission of the signal throughout the pathways (i.e., they have a similar structure that allows them to compete with the normal proteins but they have a reduced capacity, if any, to transmit the signal). The final result is that the specific pathway is partially or totally blocked and as a consequence MV replicate more easily.

Whereas the first two categories of im-MV proteins target both the innate and the specific immunosystem, the proteins included in the third category act inside the apoptotic pathways. In response to virus infection, cells switch on a complex pathway of programmed cellular dead (apoptosis) and elimination, with no or limited consequence for the surrounding tissues. Importantly, most of these im-MV proteins have been experimentally demonstrated to function as specific and critical virulence factors indispensable for the infection of MV, leading to the development of myxomatosis in European rabbits that, in fact, represents a devastating state of immune suppression of the host that usually dies for supervening bacterial infection.

The main ways to control myxomatosis in areas where MV is endemic are a combination of direct and indirect measures of prophylaxis. Basically, they include the application of biosecurity measures, in order to avoid the introduction of the infection by infected animals or by contacts with arthropod vectors, and the use of the vaccine (Stanford *et al*., 2007). The commercially available vaccines belong to the category of the live attenuated ones and are obtained by serial passage of the virus on tissue culture or in a heterologous host. Albeit they are able to induce immunity to MV for a variable time (even 9 to 10 months) that could be easily traced by using serological methods for detecting antibodies, the protection of rabbits from the infection is not fully guarantee. However, because of knowledge gained in the two last decades from research on MV (in particular on the im-MV proteins), a new family of vaccines will soon be produced and made available in a few years. Biotechnology deleted vaccines will have at least two advantages: first, to be more safe and able to induce a wider immunity since it will be well known which im-MV protein(s) have been deleted. Secondly, it will be possible to apply the DIVA strategy that is based on use of a "marker vaccine". This will allow the use of serology to ascertain if the anti-MV antibodies detected in a rabbit originated by an infection or a vaccination.

In this view, it will be necessary to develop serological assays that are able to detect specific antibodies for the single most important MV proteins. One example of these assays was developed at our laboratory where the MV serology is based on ELISA's that specifically detect the antibodies produced against the m71L protein (Cristoni *et al*., 2007). The ELISA used in routine assays is a competitive type one (Botti *et al*., 2007). A monoclonal antibodies (MAb) specific for the m71L is adsorbed at the solid phase. Sera are diluted in the microplate wells starting from the dilution 1/10 and the antigen, which are easily obtained from cells infected with MV. The competition for the binding of the antigen is between the MAb adsorbed onto the solid phase and the serum antibodies. Finally, the MAb anti m71L conjugated to the peroxidase enzyme is used to measure how much antigen is linked to the solid phase. The test has been used since 2000 in different epidemiological situations and it has been shown to be reliable and sensitive (Lavazza *et al.*, 2004a; Ferrazzi *et al.*, 2007).
Presently, more studies are in progress on in order to identify the level of antibody production with respect to the main MV proteins, included the im-MV ones.

CALICIVIRUS IN RABBITS: A REVIEW ON RHDV AND CORRELATED VIRUSES

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal acute hepatitis of wild and domestic European rabbits (*Oryctolagus cuniculus*), which was first reported in 1984 in China (Liu *et al*., 1984). It appeared in Europe in late 1986-87 causing enormous devastation to the rabbit industry, at least until the development of an inactivated vaccine and introduction of its use in prophylactic programs. RHD has been reported in over 40 countries and is presently endemic in Asia, Europe,

Central America. Outbreaks have also been recorded in Saudi Arabia and West and North Africa. RHD has been intentionally introduced in Australia and New Zealand (Cooke and Saunders, 2002), where rabbits are considered a pest, as biological control in order to keep as low as possible the level of rabbit reproduction. In 2000 and 2001, three independent outbreaks were recorded in the United States of America and more recently from 2004-2005 again in USA and in South America (Uruguay).

The European rabbit is the only species affected by RHD and no other American lagomorphs (*i.e.*, *Romerolagus diazzi*, *Lepus californicus, Sylvilagus floridanus*) have been shown to be susceptible (Gregg *et al*., 1991). As a general rule, the presence of RHD as an endemic disease is merely the consequence of the presence of steady wild and domestic European rabbit populations that makes almost impossible the goal of eradication of RHD in spite of the availability of an effective vaccine.
A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (*Lepus europaeus*) in the early 1980's in Northern Europe (Gavier-Widén and Mörner, 1991). Due to the existence of many similarities with regards to aetiology, epidemiological data and clinical-pathological features, at least initially, EBHS and RHD were considered as the same disease caused by a single agent.

The causative agent of RHD and EBHS

For some years (1984-1990), the identification and classification of RHDV have been debated and various hypotheses were put forward (i.e., parvovirus, picornavirus, calicivirus). The definitive classification of RHD (and EBHSV) as calicivirus and the subsequent definition of the new genus Lagovirus inside the *Caliciviridae* family (Figure 1) was achieved between 1991-1992, when various authors purified the non-cultivable virus from liver organ homogenates, amplified and sequenced the capsid protein, and studied its antigenic properties.

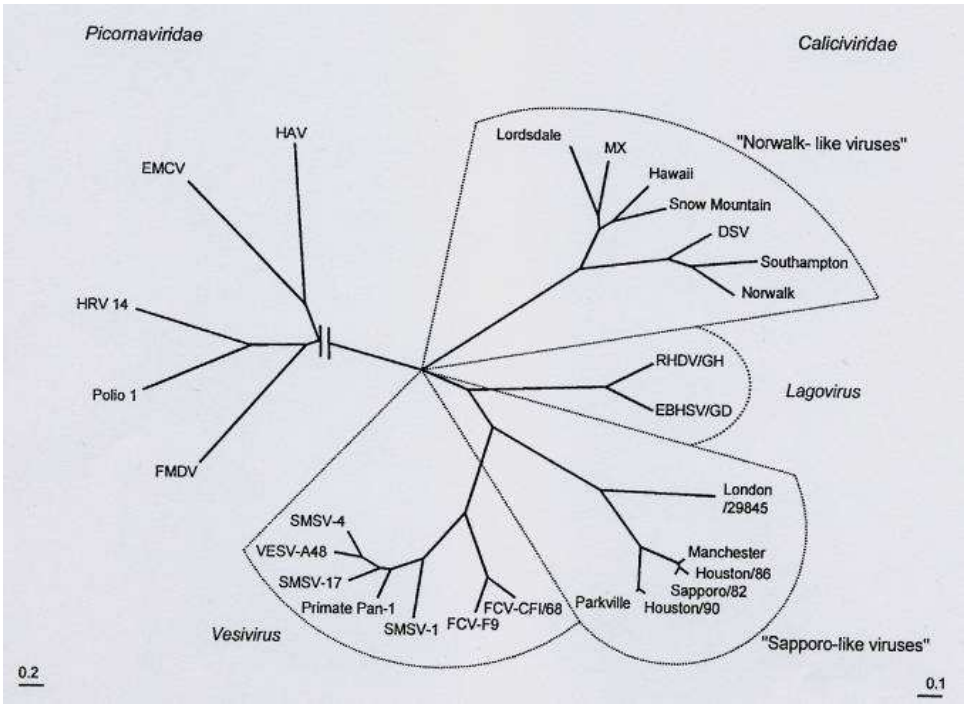

Figure 1: Classification of Caliciviridae through phylogenetic analysis of sequences of both the capsid protein and RNA polymerase

RHDV is 32 to 35 nm in diameter, has a single major capsid polypeptide (60 kDa), a positively stranded RNA genome of 7437 kb and a sub-genomic RNA of 2.2 (Capucci *et al*., 1990, 1991; Ohlinger *et al*., 1990; Parra and Prieto, 1990; Meyers *et al.*, 1991a, 1991b). The RHDV VP60 capsid protein folds in two distinct domains held together by a hinge region: the N-terminal 1 – 234 residues

constitute the inner domain and the C-terminal residues beyond 235–579 constitute the protruding domain. In the overall picture of the capsid, these domains form the inner shell and the outer shell, respectively, which is characterised by arch-like structures (Barcena *et al*., 2004). This structure also correlates with the antigenic characteristics of RHDV. In fact, the main antigenic determinants are located on the C-terminal end of the VP60 (Wirblich *et al*., 1994; Capucci *et al.*, 1995a, 1998; Schirrmaier *et al*., 1999).

Presently, it has become clear that EBHSV is not the same disease. In fact, the aetiology of EBHS remained unclear for many years until it was shown by animal experiments and electron microscopy EM analysis (Eskens and Volmer, 1989; Lavazza and Vecchi, 1989) that it was caused by a virus showing morphological characteristics indistinguishable from those of the rabbit haemorrhagic disease virus (RHDV) with biochemical features typical of the Caliciviridae family. However, significant antigenic, structural and molecular differences between the two viruses were found using RHDV-monoclonal antibodies (MAbs) (Capucci *et al*., 1991, 1995a), and cross-hybridisation and genomic sequence analysis (Wirbilch *et al*., 1994). Alignment of the RNA sequences of the EBHSV and RHDV genomes reveals 71% nucleotide identity, and amino acid alignment shows 78% identity and 87% similarity (Le Gall *et al*., 1998). Indeed, cross-infection did not occur by experimental infection of rabbits with EBHSV and hares with RHDV (Lavazza *et al*., 1996).

A second type of virus particle (s-RHDV) is commonly found as the main component in approximately 5% of RHDV-positive specimens (i.e., those taken from rabbits showing a protracted course of the disease) (Capucci *et al.*, 1991; Granzow *et al.*, 1996; Barbieri *et al.*, 1997). The main characteristics of this particle, called "smooth RHDV" (s-RHDV) are shown in Table 1. It corresponds to the inner shell of RHDV with large amounts detected, especially from 3 to 4 days post-infection, when specific anti-RHDV IgM are appearing, but only in the liver and spleen, not the bloodstream.

Table 1: Main characteristics of smooth RHDV (sRHDV) in comparison with "full" mature RHD virions (RHDV)

	RHDV	sRHDV
Diameter (nm)	32-35	25-30
Sedimentation (S)	170	145
Structural Protein (Kd)	60	28-30
HA (extract 10%)	4-8x103	neg
Infectivity (LD_{50}) (1 ml extract 10%)	105-107	neg?
Antigenicity:		
RHDV MAbs (ext. epitopes)	pos	neg
RHDV MAbs (int. epitopes)	pos	pos
EBHS MAbs (ext. epitopes)	neg	pos
αRHDV serum	pos	pos
αEBHSV serum	neg	pos

These data, in association with the finding of fragments of the VP60 having different molecular weight (41–30 kDa), during transition from RHDV to s-RHDV, led Barbieri *et al.* (1997) to conclude that the genesis of the particle is due to a degradative process that is probably the consequence of the physiological clearance of the RHDV-IgM immuno-complex formed in large amounts at the beginning of the humoral response. Therefore, the identification of this second particle in the liver of a rabbit can be considered to be a marker of the subacute/chronic form of RHD that usually evolves between 4 and 8 days post-infection, and is followed either by the death of the rabbit or, more often, by its recovery (Barbieri *et al.*, 1997).

Virus properties and resistance

RHDV is very stable and resistant in the environment; viral infectivity is not reduced by treatment with ether, chloroform or trypsin, by exposure to pH 3.0, or by heating to 50°C for 1 hour (Capucci, unpublished data). The virus survives for at least 225 days in an organ suspension kept at 4°C, 105 days in the dried state on cloth at room temperature, and 2 days at 60°C, both in organ suspension and

in the dried state (Smid *et al*., 1991). Other studies indicate that RHDV can survive in rabbit carcasses for at least 3 months, while viral particles exposed directly to environmental conditions are viable for a period less than one month (Henning *et al.*, 2005). It also retains its infectivity at low temperatures, and remains quite stable during freezing and thawing. Treatment of RHD virions at pH 11 induces the breakdown of the virions and the production of 6S VP60 subunits (Capucci, unpublished data). RHDV is inactivated by 10% sodium hydroxide, by 1.0–1.4% formaldehyde, and by 0.2–0.5% beta-propiolactone at 4°C, but such treatments do not alter the immunogenicity of the virus (Xu and Chen, 1989; Arguello Villares, 1991).

Epidemiological surveillance and virus variability

One of the most recurrent questions among researchers that study RHD and EBHS is: What is the origin of pathogenic lagovirus? In fact, it is at least outstanding that two similar diseases in two lagomorph species appeared almost instantaneously in different parts of the world. Thus, is there any relation in the emergence at the same time of two pathogenically-related but different viruses?

Most of the epidemiological studies conducted in recent years were only focused on trying to provide an answer to these questions. The extensive use of various serological methods, some of which show high sensibility, being based on Lagovirus "genus specific" reagents, permitted to reveal the presence of positive antibodies, both in rabbits and hares sampled before the first occurrence of the two diseases. Multiple data on seropositivity were gathered from wild and domestic rabbits in Australia, New Zealand, Italy, France, and United Kingdom and from hares in South America, Africa, and Europe, leading to the hypothesis of the existence of non-pathogenic RHDV-like and EBHSV-like viruses in rabbits and hares, respectively, from which originated the "new" pathogenic viruses during 1980. The factor responsible for the pathogenic shift and the molecular determinant for pathogenicity on RHDV genome have not yet been defined, but it is accepted that the RHDV-like viruses were originally "enteric" viruses, which then acquired the capacity to pass the mucosal barrier and to infect hepatic cells. How such an event occurred is not known, but it is almost certainly the outcome of a genetic mutation considering that RHDV, like all other RNA viruses, is endowed with considerable genetic variability.

This aspect and the rapid diffusion of RHDV throughout the world should have favoured a high antigenic variability. In spite of this, since the first identification of RHDV in 1984, all known viral isolates were considered to belong to one serotype. The complete sequence of geographically different RHD strains has been reported and their comparison reveals close overall homology in terms of genome sequence with few or no predicted changes in amino acid composition (differences between 2 and 5%) (Nowotny *et al.*, 1997; Le Gall *et al*., 1998). Nevertheless, isolates that exhibit temperature-dependent differences in haemoagglutinating characteristics were successively described (Capucci *et al.*, 1996a) and then a consistent genetic and antigenic RHDV variant, named RHDVa, was concurrently identified in Italy (Capucci *et al.*, 1998) and Germany (Schirrmaier *et al.*, 1999).

The RHDV variants (RHDVa and other subvariants)

The variant strain RHDVa presents amino acid changes in the surface-exposed region (aa 344–434) of the VP60 that contains the main antigenic epitopes of calicivirus, which are three times higher than in all previously sequenced RHDV isolates. It does not react in ELISA tests with the monoclonal antibody (MAb) 1H8, which is able to protect *in vivo* experimentally infected rabbits from the "classical" RHD strain, and is also less reactive with rabbit sera produced from the original RHDV isolate. However, rabbits experimentally vaccinated with the currently available RHDV vaccine were protected against challenge with RHDVa, even with a lower efficiency (Capucci *et al.*, 1998).

The production of a panel of specific MAbs was the basis for the development of specific methods in order to rapidly distinguish between outbreaks caused by RHDV and RHDVa and to enlarge the capacity to detect new possible variants. Using an ELISA test, epidemiological studies were carried out to compare the rate of diffusion of RHDV and RHDVa in Italy (Grazioli *et al.*, 2000; Lavazza *et*

al., 2004b) and elsewhere. RHDVa is present in most parts of Italy and its prevalence increased from its first reporting until present reaching values over 50% (Table 2). The highest percentages were found in those Italian regions where the most intensive rabbit production is concentrated (Lombardia, Emilia Romagna, Piemonte, Veneto and Campania).

Table 2: Total number of RHD cases observed in Italy during the last four years and relative frequency of classical (RHDV) and Variant (RHDVa) strains

Year	Tot. examined	Total RHD pos. (%)	RHDV pos. (%)	RHDVa pos. (%)
1997	n.d.	211	191 (90.5%)	20 (9.5%)
1998	n.d.	80	66 (82.5%)	14 (17.5%)
1999	n.d.	65	39 (60.0%)	26 (40.0%)
2000	252	134 (53.2%)	89 (66.4%)	45 (33.6%)
2001	136	69 (50.6%)	25 (36.2%)	44 (63.8%)
2002	203	138 (67.9%)	61 (44.2%)	77 (55.8%)
2003	226	63 (25.9%)	12 (19.0%)	51 (81.0%)
2004	209	124 (59.9%)	32 (25.8%)	92 (74.2%)
2005	192	77* (40.1%)	32 (41.6%)	40 (51.9%)
2006	171	63 (36.8%)	27 (42.8%)	36 (57.2%)
2007	406	156 (38.4%)	61 (39.1%)	95 (60.9%)

*5 (6.5%) samples not determined.

The variant has been contemporaneously identified in Germany (Schirrmaier *et al.*, 1999). It has been detected in France and on the Reunion Islands during 1999-2000, and more recent data from France indicate that it represents 10% the isolates (Le Gall, personal communication). It was identified in Malta in 2004, and it has also been reported as present in Hungary since 2003 (Matiz *et al*., 2006). All together these data suggest that RHDVa could be diffused throughout other European countries that have been experiencing the disease for many years. Outside Europe, it caused the first outbreaks of RHD in USA in spring 2000, and again it has been identified in USA on 2005. It has also caused the first outbreak in South America (Uruguay 2004-05). Taking account of the RHDV genetic sequences deposited at the NCBI databank, the presence of RHDVa in China is also evident from 1985. No data from other countries are available.

In addition to the data on the presence and diffusion of RHDVa, an epidemiological investigation led to the identification of some other RHDV isolates that presented peculiar antigenic characteristics (Capucci, unpublished data). In fact, these RHDV strains, isolated during distinct outbreaks that occurred in widely-separated areas, and at different times since 2004 to present, show different levels of reactivity using the panel of MAbs with respect to both the classical strain and the variant RHDVa. Based on their reactivity with MAbs, these strains could be considered as further and separate steps of variation of the RHDVa, possibly classified as sub-variants. Indeed the genome sequencing confirmed the existence of some genomic differences in comparison with the RHDV strains previously identified.

Epidemiological surveillance and serological surveys

As part of the epidemiological surveillance effort, serological surveys have been performed since the first occurrence of RHD in Europe in 1989 in order to determine the presence of specific anti-RHDV antibodies in wild and domestic population and to verify the efficacy of indirect prophylaxis (vaccination) in industrial rabbitries.

The standard methods employed are competition ELISA (cELISA), which is a highly specific test that measures antibodies directed against antigenic determinants on the viral external surface (binding with high avidity), and the anti-isotype ELISA (isoELISA) that enables titration of IgA, IgM and IgG (Capucci and Lavazza, 2004). The combination of the results of both methods are critical for the interpretation of field serology (Table 3) (Cooke *et al.*, 2000), considering that rabbits with titres of 1/10 in cELISA are protected from RHD and that: i) convalescent rabbits show titres of 1/640-1/20480; ii) young rabbits (<35-40 days-old) with titres 1/80-1/320; iii) vaccinated rabbits with titres of 1/160-1/320 and iv) healthy rabbits are usually negative but they can even have titres of 1/10-1/320.

It was precisely the detection of seropositivity in the sera of laboratory rabbits taken between 1975 and 1985, which is approximately ten years before the first occurrence of the disease in Europe and on farms where the disease had never been previously reported, as well as similar serological data from more laboratories (Rodak *et al*., 1990; Capucci *et al*., 1991; Trout *et al.*, 1997; Marchandeau *et al.*, 1998a, 1998b), that suggested the hypothesis of the existence in domestic rabbits of one or more "non-pathogenic" viral strains antigenically related to pathogenic RHDV.

Table 3: Summary of different immunological classes developed on the basis of cELISA, isotypes titre and body weights (from Cooke *et al.*, 2000)

	Titre*				
Class	cELISA	IgG	IgM	IgA	Notes
Negative	–	–	–	–	
Pre-existing antibodies	± (rare)	+	–	–	
Maternal antibodies	+	+	–	–	Rabbits < 1300 g
Previously infected rabbits					
Recent infection	++	++	++	++	IgM > 640
Past infection	+	+	±	±	
Re-infected rabbits	++	++	–	+	IgA > 160

* ++, high titre; +, low titre; ±, not always detected ;–, no antibodies.

The apathogenic calicivirus (RCV)

The first non-pathogenic virus related to RHDV identified in healthy rabbits was named Rabbit Calicivirus (RCV) (Capucci *et al.*, 1996b, 1997). Its existence was first suspected when we observed the spontaneous seroconversion of rabbits reared in the animal facility of our institute in the absence of signs of the disease and mortality; we then succeeded in reproducing the phenomenon under controlled conditions (Capucci *et al.*, 1995b). RCV may be considered an "enteric", non-pathogenic virus highly correlated to RHDV, perhaps its progenitor. It is able to "persistently infect" commercial rabbit farms and it works as a natural vaccine. RCV significantly differs from the previously characterised RHDV isolates because it is avirulent, replicates in the intestine at a low titre, and presents a genomic identity with RHDV of around 92%. The RCV amino acid sequence of the main capsid protein (VP60) between aa 300 and 311 is the more divergent among all the known sequences of lagovirus. Therefore, this area of the VP60, which is known to be highly variable also among different feline calicivirus isolates showing different degree of pathogenicity (Seal, 1994), could hypothetically influence the degree of pathogenicity of the caliciviruses. Experimental infection of hares with RCV failed and the antigenic data and sequence comparisons demonstrated that RCV is much more closely related to RHDV than to the EBHSV (Capucci *et al.*, 1996b).

The diffusion of RCV in different commercial rabbitries from different areas of Italy has been evaluated during five separate serological surveys by checking at slaughtering the presence of anti-RHD antibodies in non-vaccinated meat rabbits from farms with no evidence of overt RHD clinical disease. These surveys were conducted during 1999 in North Italy (Veneto and Lombardia regions), during 2002-2003 in Central and South Italy (Lazio, Campania and Basilicata regions), in 2004 and 2006-2007 in Central Italy (Marche region) and in 2007-2008 again in North Italy (Lombardia and Veneto regions). The results (Capucci *et al.*, 2004; Lavazza *et al.*, 2007; Capucci and Lavazza, unpublished data) clearly show that antibodies reactive with RHDV are present in several rabbit populations (Table 3). In particular, by using standard cELISA, we found anti-RHDV antibodies in almost 30% of controlled farms with over 80% of animals having titres ≥ 1/20 (up to 1/1280). In addition, by using anti-isotype ELISAs, clearly shown was the presence of IgA (and sometimes IgM), proving that an active infection had occurred. In addition, during the last survey it was possible to identify the viral strains by using PCR methods on faeces (Capucci *et al.*, unpublished data).

The RHDV-like viruses

The existence of non-pathogenic caliciviruses in wild rabbits was suggested (Trout *et al.*, 1997; Marchandeau *et al.*, 1998a, 1998b; O'Keefe *et al.*, 1999; Robinson *et al.*, 2001; Cooke, 2002; Cooke *et*

al., 2002) to explain the early discrepancies found in serological surveys of rabbit populations in European countries, Australia and New Zealand. RNA particles related to RHDV were shown in rabbit sera collected since 1955 in Britain, confirming that RHDV-like viruses were present in Europe for many years before the first evidence of RHD (Moss *et al.*, 2002). High antibody levels detected after RHD first began to spread through Europe but in areas where RHD had never been recorded nor suspected, provided further serological evidence that non-pathogenic strains might be present in wild European rabbit populations (Trout *et al.*, 1997; Marchandeau *et al.*, 1998a, 1998b). In addition, more recent data (Marchandeau *et al.*, 2005) provide evidence for the existence of non-protective antibodies raised against a putative RHDV-like virus.

The serological data obtained on rabbit sera in Australia and New Zealand (O'Keefe *et al.*, 1999; Cooke *et al.*, 2000, 2002; Nagesha *et al.*, 2000; Robinson *et al.*, 2001; Cooke, 2002) clearly show that antibodies reactive with RHDV were present in feral rabbit populations before the introduction of RHDV. Furthermore, the comparison of the results obtained using different ELISA systems, providing different levels of specificity but higher sensitivity indicated that the major part of these antibodies was cross-reactive antibodies, recognizing antigenic determinants buried inside the structure of the RHDV capsid (i.e., "common" epitopes considered as "group specific" in all the calicivirus of lagomorphs) (Capucci and Horner, unpublished data). Therefore, the presence and circulation of a putative non-pathogenic RHDV-like virus in feral rabbit populations being able to induce antibodies partially cross-reactive with RHDV was postulated. In addition, the serological data (average of titres found in Europe by testing with cELISA the sera of animals infected with RCV were 8 to 16 times higher than the titres found among the feral rabbit sera from New Zealand and Australia) indicated that such a putative RHDV-like virus is characterized, differently than RCV, by a consistent genetic and antigenic difference from RHDV, estimable in more than 40% of amino acid substitutions in the outer part of the VP60 (Capucci, unpublished data). The very recent isolation and identification of one of these viruses in Australia (T. Strive, CSIRO Entomology Division - Canberra, personal communication) finally confirmed this hypothesis.

The possible role of these non-pathogenic RHDV-like viruses in reducing the impact of RHD by conferring a cross-protection was also discussed (Cooke, 2002; Cooke *et al.*, 2002; Marchandeau *et al.*, 2005). The main question was whether these antibodies interfere with RHDV infection and the course of the disease, but the data obtained suggest that was not the case. In fact, there is a strong correlation between the titre in cELISA and the state of protection (i.e., animals with titres ≥1/10 are immune, but only when antibodies are specifically induced by RHDV or RCV).To the contrary, the antibodies directed towards internal determinants (cross-reactive antibodies) have little or no importance from a protective point of view; they are not neutralising and do not interfere with the RHDV infection (Cooke *et al.*, 2002; Marchandeau *et al*., 2005).

In conclusion, even if the existence of non-pathogenic RHDV-like viruses in wild and domestic rabbit populations have been proven by valid serological and virological data, their epidemiological importance remains largely unknown. Moreover some questions still need to be answered from further scientific studies. For example, are rabbits quickly building resistance to RHDV infection? Are changes in viral RNA sequences associated with virulence changes? Therefore, it will be therefore very interesting to follow the evolution in Oceania of the relationship between rabbits and the virus that cause RHD, in comparison with the previous experience of the deliberate introduction of the Myxomavirus (i.e., if a small round RNA virus, such as RHDV will evolve in less virulent strains and will select in resistant populations of rabbits as occurred for Myxomatosis virus, a large DNA virus).

VIRAL ENTERITIS OF RABBITS

Enteric diseases have an important role in the rabbit industry since they can produce severe economic losses due to mortality, growth depression and worsening of conversion index. The "multifactorial enteropathy", known also as "enteric syndrome", is the most important "conditioned" diseases, especially in relation to its productive and economic impact. It is a pathologic complex of growing

rabbits characterized by a great number of stressors and pathogens acting in synergy with a different degree of virulence (i.e., various aetiological agents (viruses, bacteria and protozoa) that can act together to cause tissue damage at the gut level, thus determining severe diarrhoea and malabsorption). Among the different pathogens that could be found in rabbits suffering from enteropathy, viruses seem to have and important but not definitive role. Viruses should not be able to induce primary episodes of high gravity but, acting as mild pathogens, they could have the capacity of becoming endemic.

In intensive rabbit breeding systems, this condition is characterised by intense genetic selection, exasperated by high productive performances, and sometimes by overpopulation and consequently high environmental load with facultative pathogens. Therefore, viruses and other low pathogenic agents (es. flagellata protozoa), can implicate a more important role for the occurrence of severe enteritis in rabbits, predisposing and aggravating secondary microbial infections. One possibility, already proposed by others, is that viruses can primarily cause damage to the intestinal mucosa, thus predisposing the attachment and replication of bacteria. In such cases it is not excluded as a dose dependant effect, as well as a transient infection and a short period of excretion, thus making possible the detection of viruses in association with the presence of *E. coli*, *Clostridium* spp, Coccidia and other protozoa. On the other hand, we can not exclude that the change in physiologic and metabolic conditions, induced to enteric level by various factors, both alimentary or not, can enhance the replication of viruses normally present at a lower concentration, permitting them to trigger a pathogenic reaction.

Rotavirus

The Group A rotavirus, a member of the *Reoviridae* family, is considered to be the main cause of acute viral gastroenteritis in different animals including rabbits (Schoeb *et al.*, 1986; Thouless *et al.*, 1996). Lapine Rotavirus (LRV) is considered only mildly pathogenic (Thouless *et al.*, 1988), but it can primarily cause enteric disease in post-weaning rabbits and be involved in the aetiology of more severe enteritis outbreaks in association with other viruses, bacteria (*E. coli*, *Clostridium* spp) and parasites. Rabbits become infected by the oro-fecal route, and the extension and the severity of the lesions are dose dependent (i.e., the consequences of the infection (microvillus degeneration, malabsorption and diarrhoea) are higher when the infectious dose is also high).

The persistence of maternal antibodies until 30 to 45 days can reduce the symptoms of the disease. Thus, until 4 to 5 weeks of age, rabbits mostly became sub-clinically infected with particle excretion for only 3 days. The LRV infection is more frequent in growing rabbits (35 to 50 days old) and is characterised by a high rate of morbidity, with non-specific clinical signs (i.e., diarrhoea, anorexia, and depression). Diarrhoeic symptoms appear at the beginning of viral excretion that lasts for 6 to 8 days, and are generally followed by constipation. Lesions observed at necropsy are not constant: catarrhal, haemorrhagic or necrotic entero-tiflitis and caecal impaction. Meat rabbits suffering from enteritis can die due to dehydration and secondary bacterial infections. In rabbits that recover from the infection, a decrease in productivity is commonly observed due to reduced absorption capacity.

A virological diagnosis can be achieved by testing faeces and intestinal contents by ELISA, including negative staining by electron microscopy (nsEM) and PCR. LRV was detected in 16.4% (Nieddu *et al.*, 2000) and 23% of post-weaning rabbits with enteric signs (Cerioli *et al.*, 2004); however, sero-epidemiological surveys have shown that most adult rabbits are seropositive for rotavirus, thus indicating that there is normally a constant circulation of low amounts of rotavirus in industrial rabbit farms (Peeters *et al.*, 1984; Di Giacomo and Thouless, 1986). The introduction of breeders of unknown origin, without application of a quarantine period, is an important risk factor. Thus, a reduction in biosecurity and hygienic activities (e.g., cleaning, disinfection, removal of litters) can lead to a dramatic increase of the environmental contamination with rotavirus.

The classification of rotavirus strains is based on the characterization of two outer capsid proteins, VP4 and VP7, the main antigenic determinants that independently elicit neutralising antibodies and induce a protective immunity response. Based on both antigenic or genetic characterization, 15 VP7

types (G types) and 26 VP4 types (P genotypes) have been recognized (Estes, 2001). A few LRV strains have been analysed in detail in early investigations. Analyses of the few strains identified in various parts of the world (Canada, USA, Japan, Italy) have revealed a substantial antigenic/genetic homogeneity of LRV's, as all the viruses analyzed so far belong to the VP7 serotype G3 (Petric *et al*., 1978; Sato *et al*., 1982; Conner *et al*., 1988; Thouless *et al.*, 1988; Ciarlet *et al*., 1997) and to the VP4 serotype P11[14] (Ciarlet *et al*., 1997; Hoshino *et al*., 2002). The epidemiological surveys carried out to investigate the distribution of the VP7 and VP4 antigenic specificities of LRVs in Italy are fully reported by Martella *et al*. (2003, 2004, 2005). Almost all the strains were characterized as P[22],G3 (Martella *et al.*, 2005), confirming the presence of the newly-recognized rotavirus P[22] VP4 allele in Italian rabbits. Only one P[14],G3 LRV strain was identified and two samples contained a mixed (P[14] + [22],G3) rotavirus infection. All the LRV strains analysed exhibited genogroup I VP6 specificity and a long dsRNA electropherotype. However, one of the P[14],G3 strains possessed a super-short pattern. Overall, these data highlight the epidemiological relevance of the P[22] LRV's in Italian rabbitries.

Coronavirus

Rabbit Coronavirus (RbCoV) is an unassigned member in the *Coronaviridae* family. It has been described as an agent of two different pathologic forms in the rabbit: a systemic disease (known as pleural effusion disease or cardiomyopaty of rabbit) and an enteric disease (Lapierre *et al*., 1980; Osterhaus *et al*., 1982). The systemic disease is characterized by fever, anorexia, leucocytosis, lymphocytopenia, anaemia, hypergammaglobulinemia, iridocyclitis, which is often followed by death. The lesions are localized to the myocardial and pleuric levels. The enteric disease shows the lesions and symptoms typical of enteritis caused by coronavirus in other species. The RbCoV replicates in small intestine with necrosis of apical villi and is followed by diarrhoea (Descoteaux *et al*., 1985; Descoteaux and Lussier, 1990). A high prevalence has been found in seroepidemiological surveys (Deeb *et al*., 1993), indicating a wide diffusion of the RbCoV in rabbitries.

Diagnosis of coronavirus could be achieved by using negative staining electron EM. The important increase of coronavirus-like positivity from our previous surveys (Nieddu *et al.*, 2000; Cerioli *et al.*, 2004) suggests the need for further improvements for studies of this agent; however, which role as either an enteric and/or systemic pathogen has not yet been completely determined. Serological surveys performed in three rabbitries, using an indirect ELISA based on the use of cross-reactive reagents for Bovine Coronavirus (BCV), indicated a widespread seroprevalence. However, by testing with a sandwich ELISA for BCV 16, samples resulted in positive cases by EM for coronavirus-like particles; we only detected a faint positivity in 6 of samples. We also tried to isolate *in vitro* the virus and to define its haemoagglutination properties: RbCoV, similarly to bovine BCV, seems to grow in the HRT18 cell line, and it haemogglutinates mouse red blood cells but not those of rabbit. In these surveys coronavirus was frequently associated with other viruses (mainly with rotavirus), accounting for 80% of its association during the period 2002-2004, so it could be possible that it can act together with viral and bacterial agents to determine post-weaning enteritis. Therefore, the pathogenic role as a cause of primary enteric disease was not evident, but the widespread nature of the virus and its high seroprevalence (100% farms, 3 to 40% rabbits) as observed by other authors suggest the possibility of subclinical infection and a probable role as an opportunistic pathogen.

Other viruses

The Rabbit Parvovirus, first described by Matsunaga *et al*. (1977), has very low pathogenicity and it is commonly isolated from the gut contents of healthy animals. It could cause very mild clinical signs (lethargy, disorexia, and depression) in experimentally infected animals and a mild to moderate enteritis in the small intestine (Matsunaga and Chino, 1981). Its primary pathogenic role is still unclear, but considering its frequency of identification, it could be important only in multiple infections when combined with other infectious agents (viruses, bacteria, and other parasites).

Some of the other viruses detected during diagnostic activity has only had a sporadic occurrence, thus their pathogenic role is probably negligible. Adenovirus has been previously reported only once (Bodon and Prohaszka, 1980). Reovirus and enterovirus have never been described as enteric agents of rabbits. However, we cannot exclude that enterovirus-like particles correspond to picobirnavirus (Gallimore *et al.,* 1993), stating that strict morphological similarities exist with this group of non-cultivable RNA viruses as identified in several species (humans, pigs, chickens, guinea pigs) including rabbits. Lusert *et al.* (1995) found that picobirnavirus was commonly excreted by 10% of rabbits without causing any symptoms or lesions. A cultivable calicivirus, *genus* vesivirus, has been recently identified from juvenile growing rabbits showing symptoms of diarrhoea (Martin-Alonso *et al*., 2005) and it was shown to be neither related to Rabbit Haemorrhagic Diseases virus (RHDV) nor to Rabbit Calicivirus (RCV).

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