IDENTIFICATION IN RABBITS AND PRELIMINARY CHARACTERIZATION OF A NON-PATHOGENIC CALICIVIRUS CORRELATED TO RABBIT HAEMORRHAGIC DISEASE VIRUS (RHDV)

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Abstract - We conducted a serological survey on 176 rabbits for anti-Rabbit Hemorrhagic Disease Virus (RHDV) antibodies, in an industrial rabbitry. We identified a calicivirus, provisionally called Rabbit Calicivirus (RCV), antigenically related to RHDV but without its pathogenicity. In particular, we observed: 1) a seroprevalence of 22.7% among young animals at weaning (31 days old); 2) a rapid increase in positive rabbits during their fattening period: 25% at 5-7dd post weaning (p.w.), 55.6% at 13-14dd p.w., 87.5% at 19-20dd p.w., 100% at 32-33dd p.w.; 3) all breeders were seropositive, 10% had a high titre (1/640-1/1280). In the latter and in young at weaning, the antibodies were class IgG. In animals at 13-14dd p.w. they were however classes IgM and IgA. In older fattening rabbits, a decrease of IgM and IgA and a contemporary increase of IgG confirmed seroconversion without any specific RHD symptoms. We isolated and characterised the RCV using RT-PCR and Western Blotting in the proximal intestine of 6 days p.w. rabbits. During two separate experimental trials, we inoculated 2 month old rabbits and hares with intestinal extracts containing RCV, and succeeded in: 1) reisolated RCV in the intestine of rabbits killed 3 days post infection (p.i.); 2) observed seroconversion from 4 days p.i.; 3) demonstrated the acquired resistance against RHDV; 4) excluded the susceptibility of hares to the infection. Finally we have discussed the epidemiology of the natural RCV infection in the rabbitry and future implications of the identification of such an non pathogenic virus.

INTRODUCTION

Rabbit Hemorrhagic Disease (RHD) is an acute and fatal disease of wild and domestic rabbits (*Oryctolagus cuniculus*) first described in China 1984 (LIU *et al.*, 1984). It has a high morbidity and mortality rate close to 90% in adult rabbits and is now widespread throughout the world. Its etiological agent (RHDV) has been well described and classified as a member of the Caliciviridae family (OHLINGER *et al.* 1990; CAPUCCI *et al.*, 1991).

In recent studies, the existence of a single highly virulent serotype of RHDV has been proposed. However many data suggest the existence of a less virulent or wholly non pathogenic correlated virus circulating in Europe for more than twenty years. Indeed, antibodies cross-reacting with RHDV have been found recently. These were in the testing sera of laboratory rabbits collected between 1975 and 1985 in the Czech Republic (RODAK *et al.*, 1990) and in certain rabbit populations, in which RHD had never occurred clinically, in the Czech Republic (RODAK *et al.*, 1990) and in Italy (SCICLUNA *et al.*, 1990). Furthermore RHDV has a close antigenic relationship with the agent of a remarkably similar hare disease, i.e. European Brown Hare Syndrome (EBHS) (CAPUCCI *et al.*, 1991; CHASEY *et al.*, 1992). Nowadays EBHS is responsible for significant losses in domestic and farmed hares all over Europe. Indeed RHDV and EBHSV, which share structural determinants, are clearly two distinctive and stable entities (WIRBLICH *et al.*, 1994). There is no proof of a direct derivation from one to the other, but the hypothesis of a common pathogen ancestor has been suggested. Until now, nobody has identified nonvirulent RHDV strains. However, in the absence of true epidemics, naturally acquired antibodies, have been reported from Switzerland (MCCULLOGH, unpublished results) and Austria (NOWOTNY and STEINBECK, 1991). Similarly, we have recently reported seroconversion, without symptoms, of rabbits reared in our animal facility and we succeeded in reproducing the phenomenon under controlled conditions (CAPUCCI *et al.*, 1995b). The origin of the agent responsible for the seroconversion appeared to be external and it was ascribed to the introduction of rabbits from a commercial supplier. Animals from this industrial rabbitry were found seronegative two months before the observed seroconversion in our facility. However these were found seropositive when tested at the same time and remained seropositive and asymptomatic for the next three months.

We therefore decided to study the evolution of the asymptomatic infection directly in this industrial rabbitry. The aims were: 1) to confirm the natural and continual occurrence of seroconversion; 2) to define at which moment it occurs and in what age of rabbit; 3) to describe the seroconversion, also in terms of classes of specific anti-RHDV immunoglobulins, found directly in the farm or in animals from the farm and reared in

isolated conditions; 4) to isolate and identify the non pathogenic RHDV-like virus by virological methods and 5) to reproduce the phenomenon by experimental inoculation of seronegative rabbits with extracts of tissues containing the non-pathogenic strain.

MATERIAL AND METHODS

Rabbitry

The industrial rabbitry has 1100 breeding cages, occupied by New Zealand White and New Zealand White crossed does. It is mainly closed, in that few bucks are only periodically brought in. About 60 bucks of different breeds are maintained and used for artificial insemination. Rabbits are sold for meat when they are about 77-82 days old. Breeders and newly-born are housed together with young meat rabbits in 2 new adjacent and closed sheds. There are six rows of battery wire-cages in each of them. In the four middle ones the does with offspring are housed, in breeding cages of flat-deck construction, until weaning (31 days). In the lateral rows there are more battery wire-cages with a multi-level design (three partially overlapping cages on both sides) where post-weaned rabbits are housed until slaughter as well as young breeders. Each row usually shelters animals of the same age. After weaning the does are moved according to a weekly cyclic rotation of the animals. A program of direct prophylaxis based on cleaning the environment, desinfection and desinfestation is systematically carried out. The last vaccination against RHD was in March 1994 and there were no subsequently vaccinations. The sanitary condition of the rabbitry was good and the mortality level of the rabbits at various ages were considered normal.

Animals

During the period January/March 1995, we collected 176 sera, principally from p.w. fattening rabbits (146), aged between 1 and 40 days p.w.. The 7 bucks tested were all born or introduced in the rabbitry after the last RHD vaccination. All the 23 does tested had littered either once or twice, they were therefore less then 10 months old (Table 1). Initially, we randomly took 36 sera for basic data on the serological situation of the farm. Afterwards, the number and type of animals to be sampled was established after the previous sampling results. We also sampled 6 groups composed of one doe and 3 offsprings at weaning from its own litter. At the end of the first phase of the survey we took another group of sera from 4 does and of just one of their own weaned offspring. These were reared in an isolation room for two months in our institute. Periodically they were serologically controlled and finally they were infected using a virulent RHDV strain. During the following months we checked the serology level of all rabbits, which were usually more than 4 months old and had been introduced into our facility from the same rabbitry. Nine months after the previous sample, another 4 groups of 5 rabbits were sampled which were respectively 0,7,14,21 days p.w., as well as a group of 10 breeding does.

Table 1 : Schedule of the samples taken

Date / Animal	11- jan Nr	19- jan Nr	26- jan Nr	02- fév Nr	09- mar Nr	17- mar Nr	Total Nr
bucks	7						7
does	13		3^		3^	4*	23
1°			9^		9^	4*	22
5- 7°	3	5			10	6	24
13- 14°			11	15	10		36
19- 20°		5	6	15			26
26°		5					5
32- 33°	4	10					14
39- 40°	9	10					19
Total	36	35	29	30	32	14	176

° for young the age is expressed as days post weaning (31days of age)
^from each doe three youngs were sampled
* from each doe one young was sampled

Serological analysis

Blood samples were collected from the rabbit's marginal auricular artery. Sera were taken and stored at -20°C until tested. A blocking ELISA was used to test rabbit sera for specific anti-RHDV antibodies (CAPUCCI *et al.*, 1991).The identification of the different classes of anti-RHDV immunoglobulins (Ig) present in the sera was made after producing anti isotype monoclonal antibodies (MAbs) and after evaluating their activity and type by ELISA on different coated antigens (purified rabbit Ig and various types of sera) (NARDIN and CAPUCCI, unpublished data). Two different types of ELISA reaction were used to find respectively IgG and IgM-IgA: 1) the quantification of the specific IgG required the entrapping of the RHDV antigen with an anti-RHDV MAb absorbed on a microplate. The sera were then serially diluted and the IgG bonded to the virus were revealed by a HRP-conjugated MAb which is anti-rabbit IgG; 2) to find the IgM and IgA, two different MAbs, anti-rabbit IgM and anti-IgA respectively, were directly coated on a microplate on which the sera were diluted. Then the previously tittered RHDV antigen was added and shown up with an anti-RHDV MAb which was HRP-conjugated.

Virological analysis

At the end of the first phase of the survey, when the probable phase of infection with the RHDV-like agent had already been determined, we took 6 rabbits at 6 days p.w.and brought them to the laboratory. Here they were humanly killed, necropsied and portions of their organs were submitted for viral identification. We took samples of their organs and tissue where RHDV usually multiplies: i.e. the liver, the kidney, the epithelium of the nasal mucosa as well as the intestine. This latter was taken because some of the biochemical characteristics of RHDV, i.e. extreme resistance to low pH. and protheolitical enzymes, are typical of enteric viruses which develop in the intestine. We referred to the existing correlation of RHDV and to parallel studies of EBHSV for the choice of methods and reagents. In fact, even given that the EBHSV correlation is only partial, the use of RHDV specific reagents has proved to be fundamental (CAPUCCI *et al.*, 1991; WIRBLICH *et al.*, 1994). If we presume that the quantity of virus present was minimal due to the lack of a current disease, we used methods capable of revealing even a minimum quantity of the virus, while at the same time retaining a high level of specificity. We excluded using cell culture methods given that RHDV do not propagate *in vitro*. Instead we used three principal techniques: 1) Negative staining Electron Microscopy (EM); 2) Western Blotting (WB) and 3) Reverse Transcription Polymerase Chain Reaction (RT-PCR). The EM and WB protocols have been already previously described (CAPUCCI et al, 1991; CAPUCCI et al, 1995a). RT-PCR reaction was as follows: tissue fragments from 1 to 5mg. were ground with 4M guanidium isothiocyanate in a ratio of 1/5 (wt/vol). Then RNA was extracted according to the method described by CHOMZINSKY *et al.* (1987). Reverse transcription and PCR reaction were then performed on 10/30 µg of RNA extracted from all the rabbit organs, essentially following the conditions described by MEYER et al (1994) with the exception of the KCl concentration used in the amplification reaction (75 mM. instead of 4 mM.). Sequence and RHDV genome positions (ROSSI *et al.*, unpublished results) of the oligonucleotide primers used for reverse transcription (RT) and subsequent cDNA amplification of polymerase gene were as follows: Dir. Primer: 4510-4530: $^{5'}$GACTACTCAAAGTGGGACTCC$^{3'}$ - Rev. Primer. 4832-4850: $^{5'}$TCGGCGTCATGGCATACACG$^{3'}$

Experimental Infections

We carried out two experimental trials. During the first, two pairs of 2 month old seronegative rabbits were infected with the intestinal contents of two of the six rabbits taken from the rabbitry and which were initially positive to RT-PCR. A 1cc dose of this extract was inoculated half intranasally and half intramuscularly. The pair of rabbits were housed in different cages together with a control rabbit. Another two controls were housed in cages placed on the opposite wall of the room, 2.5m away. One of the two infected rabbits in each of the cages was killed 3dd post inoculation (p.i). and their organs were virologically examined. The other inoculated rabbits and the controls were serologically tested a number of times and 30dd p.i. they were infected with virulent RHDV. In the second experiment, we infected 6 rabbits intranasally, together with 4 EBSHV-seronegative hares. The serological checks were carried out every day and the rabbits were killed and virologically examined 3, 5 and 7dd p.i.. Two of the 4 hares were killed 6dd p.i. and virologically examined, while the other two were serologically checked a number of times and after 30 days were challenged with EBHSV.

Table 2 :Serological results

Titre	neg		<1/10	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	pos	Total
Animal	Nr	%	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	%	Nr
bucks	0	0,0		1	1	2	2	1				100	7
does	0	0,0		2	2	4	7	3	2	1	2	100	23
1°	17	77,3			1		4					22,7	22
5-7°	18	75,0	1	1	3			1				25,0	24
13-14°	16	44,4	5	2	3	2	4	4				55,6	36
19-20°	3	11,5	1	1	2		2	10	5	2		87,5	26
25°	1	20,0					1	2			1	80,0	5
32-33°	0	0,0				1	3	5	4		1	100	14
39-40°	1	0,0				3	3	9	3			100	19
Total	56	31,8	7	7	12	12	26	35	14	3	4	68,2	176

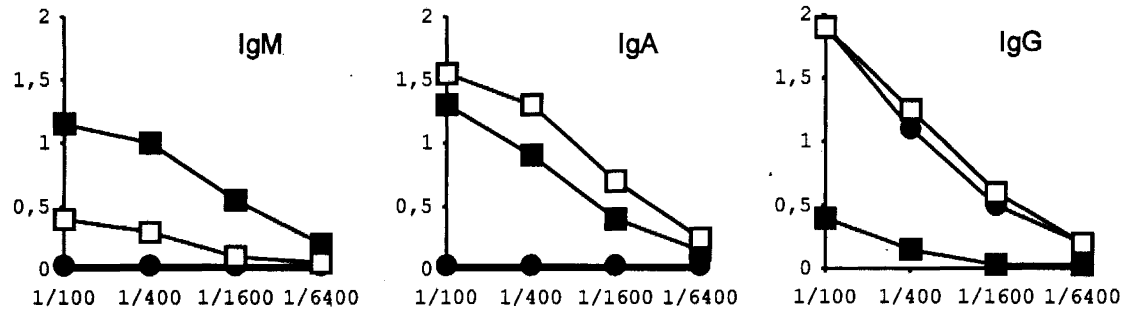
° for young the age is expressed as days post weaning (31days of age)

RESULTS

The serological results from the 176 sera taken are summarized in Table 2. All the breeders had positive anti-RHDV antibody titre, ranging from 1/10 to 1/1280, with a prevalence between 1/40 and 1/320. There was a very different situation among fattening rabbits and we noted that the presence of anti-RHDV antibodies in their sera was directly related to age. In particular, weaned animals showed a slightly inferior rate of seropositivity (22.7%) than animals 5-7dd p.w. (25%). From this point on the seropositivity increased progressively reaching 100%, 32-33dd p.w. Furthermore while the titre for animals 13-14dd p.w. (55.6%

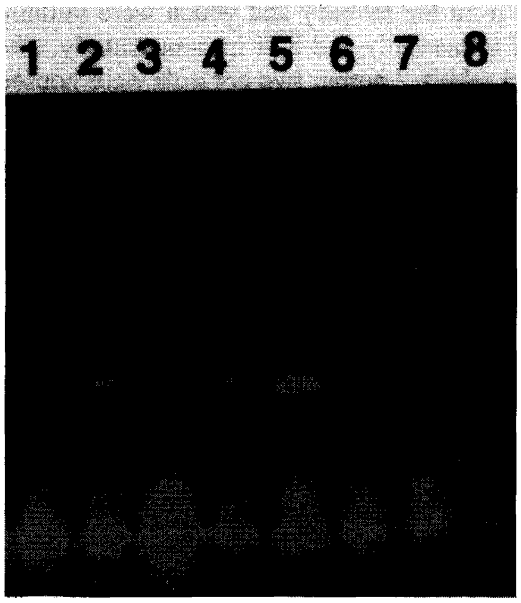
positive) was between 1/10 e 1/160, for those 19-20dd p.w. (87.5% positive) there were higher titres (up to 1/640). Finally animals already weaned for more than 20 days were all positive with a titre between 1/40 and 1/1280, with only 2 exceptions. Subsequent controls on the 4 month old rabbits introduced into our institute, were found to be positive with medium-low titres (1/80-1/160). The results from the controls carried out 9 months later, confirmed the progress of fattening rabbits and the positivity of breeding does (data not shown).
We studied the RHDV serological profile found in rabbits at weaning and fattening rabbits in respect to subclasses of antibodies (Figure 1). We noted that rabbits at weaning (●) had only IgG antibodies, while those 13-14dd p.w. had clearly both IgM and IgA (■). Finally there was a fall in IgM and a net increase of IgG in rabbits 19-20dd p.w. (□) i.e. who had passed more time from the moment of infection.

Figure 1 : RHDV serological profile for individual subclasses of antibodies



A comparative analysis of the anti-RHDV serological results showed a direct correlation in the titre of 6 breeding does and three of their young from respective litters. In particular 3 young were seropositive (1/80) whose mother had a high viral rate (1/1280), while the 3 young from mothers with a low titre (1/40-1/80) were negative. This was confirmed by serological monitoring on four young of four litters. These were taken away from the rabbitry when they were weaned and were subsequently reared in isolation. Two young from 2 mothers with medium-low titres (1/80-1/120) were negative, whereas the antibodies persisted for 12 days for those born from mothers with medium-high titres (1/320-1/1280). Furthermore both of these groups of rabbits, did not seroconvert and all died when exposed to RHDV. This was because they were reared in isolation, in contrast to the original rabbitry.

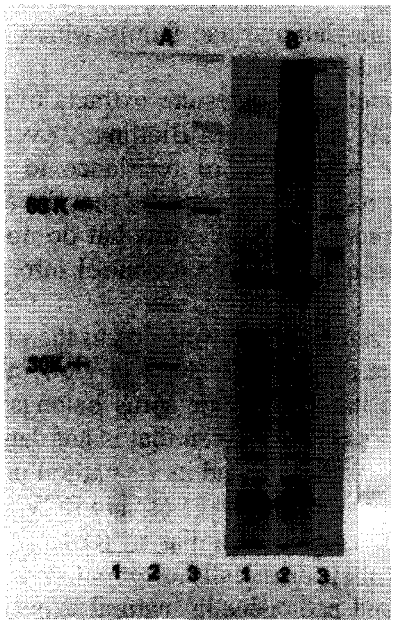
Figure 2: Direct research on the virus in the fattening rabbits by using RT-PCR



The results of the RT-PCR carried out on the intestines of six animals taken from the rabbitry at an age of 6 days p.w. are numbered progressively from 1 to 8. From the electrophoretic analysis of the RT-PCR products, there was a band in samples 2, 4 and 5 which was not in the controls 7 and 8. This former had a similar molecular weight as that expected for the length of the fragment chosen for amplification. The higher molecular weight of another band in samples 2, 5 and 7 was due to a non-specific amplification which did not correlate to the virus.

The final virological data are shown in Fig. 2 and 3. Three of the 6 day old fattening animals were positive to the RT-PCR carried out on the intestine (Fig. 2), with one case on the limits of being positive also for the liver.
We identified a protein of a similar molecular weight to the structural protein of RHDV in the intestine of two out of three rabbits, by Western Blotting (Fig. 3). This was achieved using both a monoclonal antibody produced for RHDV and anti-RHDV hyperimmune serum from convalescing animals. On the other hand, we were unable to visualize the virus by EM which is a clear indication of the very small quantity of the virus.
The first experimental infection consisted of 6 two month old rabbits with various contact tests. We were able to identify the non-pathogenic virus in the intestine of 2 of the inoculated animals, which were killed 3dd p.i., as well as an antibody reaction from day 4 p.i.. This was both in the infected animals and the controls, which had survived a subsequent RHDV pathogenic infection. The controls in the cages 2.5 metres away remained seronegative and died when exposed to the virulent RHDV. Similar results were found in the second experiment. The non-pathogenic virus was found in the rabbits killed 3 and 5dd p.i. and seroconversion was noted 5 and 7dd p.i.. The same experiment carried out on 4 EBHSV seronegative hares did not

Figure 3: Virological test using Western Blotting



The Western Blotting analysis was carried out on samples 1 and 5, which had been respectively negative and positive for PCR. These were placed in line 1 and 2 while the BS89 strain of the RHDV virus was used as a positive control in line 3. An anti-RHDV (MAb 5D1) monoclonal antibody was used in A as an immunological reagent to identify the VP60 of the viruses after electrophoretic separation. Similarly the serum of a rabbit convalescing from RHDV was used in B. A protein with a similar molecular weight to RHDV (in line 3) was found in sample 5 in line 2, but not in line 1 using the MAb 5D1, thus confirming the PCR data. The same results came from the rabbit serum in B. If we compare samples in line 1 and 2, we can say that the majority of bands are non-specific, with the exception of the 60kd also present in 2 and 3 but not in 1. This corresponded to the protein of the capsid of the virus.

show any seroconversion. Nor was it possible to identify the virus in the intestine of the two animals killed at the same time as the greatest multiplication of the virus in the rabbits.

DISCUSSION

The principle results of the seroepidemiological study carried out in the rabbitry have been outlined here: 1) 22.7% of the animals were seropositive when weaned; 2) there was a rapid increase of seropositivity with the rabbits became fattening animals; 3) all the breeders were seropositive, with 10% with a particularly high titre.

For the weaned animals (31 days old), we made four observations which showed that the antibodies came from the mothers: 1) the antibodies appeared to be entirely from the IgG class; 2) when the samples were taken both from the mothers and their month old litters, there was a clear correlation between the mothers and their litters; 3) the serological tests of 4 seropositive animals at weaning which had been taken away from the rabbitry and isolated, showed a rapid decrease of antibodies. All these animals became seronegative in less than 3 weeks. In the following two months their anti-RHDV titre remained negative and they died when exposed to RHDV; 4) the serological analysis of the breeders showed an anti-RHDV titre equal or superior to 1/640 in about 10% of subjects. Therefore the 22.7% of rabbits with passive antibodies at weaning is acceptable. However the origin of the antibodies is different in 55.6% and 87.5% of rabbits 2 and 3 weeks p.i. respectively That this is due to an infection is shown by: 1) the big increase of the positive percentage within a few days; 2) the antibodies were IgM and IgA while IgG antibodies only appeared later 3) the titre increased with time, from average levels of 1/20 in the first week to 1/160-1/320 in the fourth; 4) the continued existence after 6 months, of a positive antibody titre within individual animals even with no reinfection. The correlation of the infectious agent with RHDV was equally evident. Indeed, these results were similar to animals which had vaccinated for RHDV. On the other hand, the total absence of clinical signs and mortality within the rabbitry exclude the fact that RHDV was the infectious agent. The most realistic hypothesis to explain why the majority of animals are infected only when they pass to the fattening stage must be that the virus is not present in the breeding-cages or is there only occasionally. Therefore the young do not become infected due to the lack of contact with the infecting agent. This could be due principally to the fact that the breeders are 100% positive and therefore immune to the virus. Even if this latter is occasionally present, it cannot multiply at such a level as to create a general infection. This hypothesis is supported by previous experiments we made in our institute. Individual animals with the active infection, transmitted it only over limited periods of a few days and only to animals in adjacent cages (CAPUCCI *et al.*, 1995b). It should be added that litters from mothers even with medium titres, could be unreceptive on the first two weeks due to maternal antibodies. On the contrary, in the three-tier cages for fattening, the weaned animals which are added are generally negative. These contract the infection and therefore spread the non-pathogenic virus into the external environment. The infectious nature of the virus is thus enhanced both qualitatively and quantitatively. Given that there is a regular weekly intake of negative animals into this area, the virus can therefore perpetuate itself indefinitely. This is clearly shown within the rabbitry studied here, which became seropositive about one and a half years ago and still is. The fact that all the breeders are positive at about 10 months from their last vaccination can be explained given that the breeding is carried out by animals already positive when fattened. The high titres (1/640-1/1280), found in about 10% of the subjects could be due to occasional reinfections of the non pathogenic virus whenever animals are temporarily placed in cages adjacent to fattening rabbits. The virus can partially multiply causing the classical

« boster » effect, probably in those animals where the antibody rate has almost disappeared with time. This causes a higher titre of antibodies than the primary infection.
The virological results taken as a whole (positive RT-PCR, the presence of capsid protein of a weight of 60kD, antigenically similar to RHDV) allow us to conclude that a virus of the Caliciviridae family which closely correlates to RHDV was found in the intestine of rabbits from the rabbitry.
Carrying out two separate experimental infections in seronegative animals with intestine extracts of rabbits positive to RT-PCR we were able to: 1) reisolate the virus using RT-PCR and Western Blotting; 2) verify the rabbits' conversion from RHDV negative to positive; 3) show their acquisition of resistance to RHDV infection; 4) exclude the possibility that the identified virus also affects hares. If we consider that the animals infected were two months old (rabbits less than circa 35-40 days contract the RHDV virus but do not show signs of the disease) and that the seronegative controls died when subsequently given a control infection of RHDV, these data further indicate that the virus is non pathogenic.
The research we carried out directly in the rabbitry showed that RHDV seropositivity was due to the presence of a virus antigenically correlated to RHDV, but radically different from a phenotype point of view in that it does not cause disease. On the base of our data it is evident that the non-pathogenic virus belongs to the Caliviridae family. We could preliminarily call this virus Rabbit Calicivirus (RCV) given that is not dangerous unlike RHDV, and that usually the name of a virus derives from the species it was found in. Its natural presence seems to predate RHDV, to which it could even be its « parent » Future studies on other possible RCV isolates will be able to say if there are genetically different viral strains which are more similar to RHDV. Just as interesting will be to establish the genomic differences responsible for their drastically different end results. These two viruses, together with EBHSV could be a valid virological and pathogenetic 'natural experimental model'. This could also be useful as a comparative model for the study of human hepatitis. Finally, on a practical level, we should establish whether RCV can be used as a natural vaccine against RHDV. In this case we must find out how the infection works and above all is life span, according to the type of rabbitry as well as the absolute non-pathogenic nature of the virus.

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Identificazione nel coniglio e caratterizzazione preliminare di un calicivirus apatogeno correlato al virus della malattia emorragica virale (RHDV) - E' stata condotta un'indagine sierologica per anticorpi anti-virus della malattia virale emorragica (RHDV) su 176 conigli di un allevamento industriale. Sulla base dei dati ottenuti è stato possibile identificare un calicivirus, provvisoriamente denominato Rabbit calicivirus (RCV), antigenicamente correlato a RHDV ma privo di virulenza. In particolare è stato osservato: 1) una sieroprevalenza del 22,7% tra i conigli allo svezzamento (31gg di età); 2) un repentino aumento della sieroprevalenza tra i conigli all'ingrasso: 25% a 5-7 gg dopo lo svezzamento, 55,6% dopo 13-14gg, 87,5% dopo 19-20gg, 100% dopo 32-33gg; 3) tutti i riproduttori erano sieropositivi e fra questi un 10% ad alto titolo (1/640-1/1280). Nei riproduttori e nei giovani allo svezzamento le immunoglobuline erano di classe G, mentre negli svezzati da 13-14gg erano di classe M ed A. Nei conigli in fase avanzata di ingrasso, il calo delle IgA ed IgM, cui si accompagnava un aumento delle IgG, confermava l'avvenuta infezione virale in assenza di sintomi tipici riferibili a RHD. RCV è stato inoltre isolato dall'intestino prossimale di coniglietti svezzati da 6 gg e caratterizzato mediante utilizzo di RT-PCR e Western Blotting. In due distinte prove sperimentali, conigli e lepri sieronegativi sono stati infettati con estratti contenenti RCV ed è così stato possibile: 1) reisolare di RCV nell'intestino di conigli soppressi dopo 3gg dall'infezione; 2) osservare una sieroconversione a partire da 4gg dall'infezione; 3) dimostrare l'acquisita resistenza specifica verso challenge con RHDV; 4) escludere la suscettibilità delle lepri all'infezione con RCV.
Sono stati infine discussi l'andamento epidemiologico dell'infezione da RCV nell'allevamento e le future implicazioni derivanti dall'identificazione di tale agente apatogeno.