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Original article

Serological evidence for a non-protective RHDV-like virus

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Abstract – The data were recorded during a Rabbit haemorrhagic disease outbreak that occurred in France in 2001 in a wild population of rabbits that we have been monitoring since 2000. These data suggested the existence of non-protective antibodies due to a putative RHDV-like virus. Twenty-one blood and 22 liver samples were taken from the 26 corpses of recently dead rabbits that were found. RHDV was found in all liver samples. A first screening for RHD antibodies, carried out using an ELISA based on the detection of VP60-RHDV antigen, showed that 20 of the rabbits were seropositive. Moreover, we determined antibody titres for 13 of these 20 seropositive samples. All were ≥ 1/400. Such titres normally indicate antibody levels sufficient to confer protection to all known RHDV or RHDV-like strains. For 16 samples, we determined whether these rabbits had died of a chronic or an acute form of the disease, by employing monoclonal antibody (Mabs) – based differential ELISA. All had died of an acute form of RHD. Because the antibodies detected by this VP60-ELISA test are known to appear 5–6 days after infection and since acute RHD generally kills the rabbits 2–3 days after infection, we assumed that the detected antibodies must have been present before the exposure to the virus that killed these rabbits. A second detection of antibodies was made with Mabs that are specific for RHDV. The results were negative, showing that the antibodies detected with the VP60 ELISA test were not specific for RHDV. We sequenced a portion of the VP60 gene of viruses isolated in 17 rabbits. All RHDV isolates were very similar to the RHDV strains commonly isolated in France during this period, suggesting that this viral strain was not a putative variant that is not neutralised by antibodies. Therefore we conclude that the detected antibodies were probably due to a RHDV-like virus that induces the production of detectable but non-protective antibodies.

rabbit / RHD / antibodies / RT-PCR / RHDV-like virus

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1. INTRODUCTION

Rabbit haemorrhagic disease (RHD) was first described in China in 1984 [19] and spread throughout Europe from 1986 on [4, 24]. The causative agent of RHD is a calicivirus specific for the European rabbit *Oryctolagus cuniculus* [28] that has subsequently been completely sequenced [23, 31]. For a long time only one serotype of Rabbit haemorrhagic disease virus (RHDV) had been described. But recently an antigenic variant was identified in Italy and Germany [9, 34]. Given the genetic and antigenic differences between the variant and the original RHDV strains, the variants were considered to be a distinct subtype, designated "RHDVa" [9].

The initial impact of RHD on free-living populations is generally large since mortalities of up to 90% have been recorded in Europe and in Australia [21, 26, 36]. Antibodies against RHD were detected in sera collected in Europe between 1975 and 1987, showing that RHDV-like viruses were already present, but simply had not been detected before the first evidence of the disease [33]. More recent serological data suggest that non-pathogenic strains may usually be present in wild European rabbit populations, because high antibody levels have been detected even where RHD had never been recorded or suspected [22, 35]. Such non-pathogenic strains are also suspected to occur in Australia and New Zealand, where RHD was introduced in 1995 and 1997, respectively as a control agent of rabbit populations [14, 27, 29, 32]. The only known non-pathogenic calicivirus closely related to RHDV, called Rabbit calicivirus (RCV), was identified in the intestine of domestic rabbits in Italy [7, 8]. RCV can be distinguished from the RHDV by its tissue tropism, viral titre and the sequence of the capsid protein.

The antibodies against RCV and the putative non-pathogenic RHDV strains protect against RHD, although the conferred protection seems to be lower with some of these putative non-pathogenic strains [32]. Therefore, the possible role of these non-pathogenic RHDV strains in reducing the impact of RHD has been discussed by several authors [3, 14, 37]. More generally, their existence raises the question of the competition between the different strains and of the role of non-pathogenic strains in the epidemiology of RHD [37]. A recent study carried out in Britain showed that RNA particles related to RHDV have been present in the sera collected since 1955 [25], confirming that RHDV-like viruses were present in Europe a long time before the first evidence of RHD. Since some of these RNA particles were identified in sera collected in healthy rabbits and were more closely related to the RHDV strains than to RCV, it seems that there may be high natural variability among strains of RHDV-like viruses.

In this paper we report data recorded during a RHDV outbreak that occurred in 2001 in a free-living population of rabbits monitored as part of a long-term study carried out in western France. We collected dead rabbits that were analysed to determine the cause of their death and their serological status at the time of their death. Our data provide evidence for the existence of non-protective antibodies raised against a putative RHDV-like virus.

2. MATERIALS AND METHODS

2.1. Study area

The study took place in Cerizay (0° 40' W, 46° 49' N) in western France (Department of Deux-Sèvres). The landscape is characterised by mixed farming and hedgerows. The climate is oceanic with a continental influence. Mean annual rainfall is 780 mm and mean annual temperature is 11.1 °C. The 110-ha study area is managed for rabbit hunting. A free-living population has been established in artificial warrens. This rabbit population and its habitat have been intensively monitored since 2000 as part of a research programme on the dynamics of fragmented rabbit populations. The rabbit

density is about 5 rabbits/ha before reproduction. The RHD outbreak described in this paper was the first that occurred in this area.

2.2. Monitoring of the population

Rabbits were caught in wire cage traps and individually marked. At each capture, we also weighed each rabbit and took blood sample on a strip of blotting paper [10, 15]. We trapped rabbits every five weeks to monitor their levels of immunity to RHD during the year. When dead rabbits were found, full post-mortem analyses were performed to determine their cause of death.

2.3. Antibody detection

Blood samples were initially screened to detect antibodies using an ELISA based on the detection of VP60-RHDV antigen [16]. Briefly, two 6 mm-diameter discs of blotting paper were rehydrated in 100 µL of phosphate-buffered saline (PBS) on a 96-well microplate and stored at 4 °C overnight. The eluate was then used directly in serological tests as an equivalent of a 1/20 dilution of fresh serum. ELISA plate wells (Becton Dickinson, Falcon Probind, Meylan, France) were coated with 1 µg of recombinant baculovirus-purified VP60. RHDV antibody binding was visualised by incubating the plate wells with alkaline phosphatase antibody conjugate (Sigma Chemical, Saint Louis, Missouri, USA) at 37 °C for 60 min. The absorbance of each sample was measured at a wavelength of 405 nm with a spectrophotometer 15 min after the addition of the substrate solution (pNPP in 10% diethanolamine, pH 9.8) (Sigma Chemical, St. Louis, Missouri, USA). The eluate sample titre was expressed as the inverse of the highest dilution for which the optical density was greater than three times the optical density of the negative serum standard. Titres ≥ 100 were considered to be positive. As mentioned, all blood samples were initially tested to determine if the rabbits were seropositive, and only the samples for which we had enough blood were analysed further to determine the antibody titre. However, this technique does not enable the determination of the origin of these antibodies because it does not distinguish antibodies due to RHDV infection from antibodies due to RHDV-like virus infections. Therefore, an additional test was made to detect the presence of specific RHD antibodies. Because there was insufficient serum remaining after the first test, we tested the liver homogenate of 16 of the rabbits with ELISA tests that detect anti-RHD isotypes IgM, IgA and IgG directly in organ extracts. These ELISA use specific anti-rabbit IgM, IgA and IgG monoclonal antibodies (Mabs) and the methods employed are fully described elsewhere [5, 13].

In addition, blood samples were examined for myxoma antibodies with an ELISA test fully described elsewhere [10, 15].

2.4. Post-mortem viral diagnosis

When RHD was suspected to be the cause of death, liver samples were tested for the presence of RHDV with HA tests [21]. For 16 samples, we also determined whether the disease was acute or chronic by calculating the amount of virus and the level of viral degradation of RHDV in the liver samples [6]. In fact, from previous studies it is known that the presence of degraded viral particles (small, smooth RHDV particles called s-RHDV) 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 [1]. The method employed is a sandwich ELISA [5] based on the use of a panel of Mabs that recognise different epitopes. More precisely, the presence of degraded s-RHDV is shown by the combination of the positive reaction with Mabs that recognise internal epitopes, normally buried inside the virus but exposed after viral degradation, and the lack of reactivity with external, specific Mabs to neutralising epitopes. Moreover, this differential sandwich ELISA can discriminate between a

classical RHDV strain and "RHDVa". Such data are obtained by the use of two specific Mabs that recognise the same antigenic determinant responsible for in vivo neutralisation exposed on the surface of a classical RHDV strain (Mab 1H8) and "RHDVa" (Mab 3b12) respectively [9]. This sandwich ELISA test has been fully described elsewhere [5, 9].

2.5. Viral sequence analysis

When RHDV was established as the cause of death, the partial or complete sequences of the capsid protein (VP60) gene of different isolates were determined to compare them with the known RHDV or RCV strains. Twenty-two liver samples were analysed and the sequences of 17 viruses were characterised. For this purpose, RNA was extracted and purified from 100 µL of liver exudate obtained after thawing by an immunocapture assay [17] or with the RNeasy Kit (Qiagen, Venlo, The Netherlands). Then, a 559 bp fragment from the 3' end of the gene was amplified by RT-PCR with RHDVAU and RHDVAL degenerated primers and for two isolates, the complete VP60 encoding sequence was amplified [18]. The amplified products were analysed by conventional electrophoresis on agarose gel and purified (Geneclean II kit, Bio 101, Qbiogene, Montreal, Canada). The DNA sequences were determined by sequencing in both senses on an automatic DNA sequencer ABI 373XL (Applied Biosystems, Foster City, California, USA) with the M13 forward and reverse universal primers or specific primers [18].

We sequenced a portion of the VP60 gene of viruses isolated in 17 rabbits and aligned the sequences with those of the homologous region from the following French RHDV isolates: the reference French RHDV strain "SD89" collected in 1989, one isolate "00–13" representative of the new genogroup (G5) including the recent isolates collected since 1998 [18] and four isolates collected in autumn 2001 in western France ("01–66", "01–67", "01–68" and "01–75") and clustered in the same genogroup. We also aligned the RCV and the sequences of a German and two French RHDV antigenic variants "RHDVa" ("Triptis", "99–05" and "01–38", respectively).

Multiple sequence alignments were generated by the CLUSTAL W method and phylogenetic analysis was performed using the Phylogenetic interference package (Phylip) using the Infobiogen web site (http://www.infobiogen.fr). Phylogenetic relationships were inferred by the Neighbor-joining method. Bootstrap support percentages were calculated for each node of the tree by the Seqboot procedure (100 replicates). The tree was plotted using TREEVIEW [30].

2.6. Study materials

An outbreak of RHD occurred in the autumn of 2001. Twenty-six freshly dead rabbits were found between 20 October and 28 November. Among them, 25 were juveniles, with a weight ranging from 390 g to 1440 g (Tab. I). Data on growth rates recorded in France [20], indicate that two-month-old rabbits weigh about 450–500 g. Therefore, because the smallest juvenile (390 g) had reached 520 g before it died, all juveniles were probably more than two months old at the time of death. Six rabbits had been caught earlier, shortly before their death, and we noticed that two of them had lost weight before dying, but without showing any sign of myxomatosis. One was an adult (CY 121) that weighed 1510 g on 30 August but only 1090 g on 28 October. The other was a juvenile (CY 140) that weighed 520 g on 4 October and 390 g on 28 October (Tab. II). In all, 21 blood samples and 22 liver samples were collected.

3. RESULTS

RHDV was found in all 22 liver samples, strongly indicating RHD as the cause of death for all these rabbits. Twenty rabbits were seropositive using the VP60-RHDV ELISA test, one being doubtful. For 13 of

Table I. Age, weight and serological data recorded on the dead rabbits. A: adult; J: juvenile; +: seropositive; –: seronegative; n.s.: not sampled; n.d.: not determined.

Rabbit	Age	Weight at death (g)	VP60-RHDV ELISA	
			RHD antibodies	Titre
CYM 63	J	710	+	1/1 600
CYM63B	J	1180	+	1/1 600
CYM64	J	840	n.s.	
CYM65	J	730	+	1/6 400
CYM66	J	1230	+	1/6 400
CYM67	J	1170	n.s	
CYM68	J	800	+	n.d.
CYM69	J	1200	n.s	
CYM70	J	1160	+	1/3 200
CYM71	J	*	n.s	
CYM72	J	1180	+	n.d.
CYM73	J	1440	+	1/800
CYM74	J	1440	+	n.d.
CYM75	J	1280	+	1/800
CYM77	J	1400	+	1/3 200
CYM78	J	1000	+	n.d.
CYM80	J	1170	+	1/400
CYM81	J	1330	+	n.d.
CYM82	J	1300	+	1/6 400
CYM83	J	1200	+	n.d.
CY109	J	1220	+	1/12 800
CY121	J	1090	n.s	
CY124	A	1030	+	1/25 000
CY126	J	1200	+	1/64 000
CY137	J	620	+	n.d.
CY140	J	390	+/–	n.d.

* Corpse partly scavenged.

them, the titre was determined: all were ≥ 1/400 (Tab. I). Blood samples had been taken from five of the six rabbits that had been caught in cage traps shortly before their death. Four were seropositive on the basis of the VP60-RHDV ELISA at the date of their previous capture, but the youngest rabbit was seronegative (Tab. II). The status of the sixth rabbit, also a juvenile, was not determined. None of the 25 entire dead rabbits, the 26th had been partly scavenged, showed signs of myxomatosis. Neither did any of the 6 rabbits that had been caught shortly before their death show any sign of myxomatosis when captured. Moreover, two of them were seronegative and did not seroconvert against the myxoma virus between their last capture and their death, indicating that they had not been exposed to the myxoma virus.

We tested 16 liver samples to determine whether the rabbits had been affected by an acute or a chronic form of RHD. In the latter case, the virus that killed them might be responsible for the detected antibodies. In all these samples, even though the total amount of virus in each liver was variable (high in 8 samples, medium in 3 samples and low in 5 samples) the level of viral degradation was around 20–30%, which is indicative of an acute form of the disease. A further indication that the disease in all tested rabbits was acute was indirectly confirmed by the results of the anti-isotype ELISA tests, performed on liver homogenates. These tests indicate that none of the samples presented detectable levels of IgG, IgA or IgM. If the rabbits had survived for five days, as expected with a chronic form of the disease, we would have expected low levels of IgM antibodies to be present.

Among the sequences of the 17 RHDV isolates studied in this work, several were identical and the others differed only by a few nucleotides, confirming that all the isolates belonged to the same outbreak. The phylogenetic analysis revealed that all RHDV isolates were clustered in genogroup G5 indicating that they were very similar to the RHDV strains commonly isolated in France since 1998. Figure 1 shows the phylogenetic tree obtained with four of the 17 sequences analysed with nucleotidic divergences. To confirm these results, a phylogenetic analysis was made with the complete VP60 encoding sequences of two isolates ("ONC/CYM68" and "ONC/CYM74"). It gave a

Table II. Serological data, obtained with VP60-RHDV ELISA, recorded on six rabbits that had been caught a few weeks before death. A: adult; J: juvenile; T: time elapsed between the last capture and the death; +: seropositive; –: seronegative; n.d.: not determined.

Rabbit	Age	Last capture before death		T	Death		
		Weight (g)	RHD antibodies		Weight (g)	RHD antibodies	Titre
CY109	J	625	+	107 days	1220	+	1/12 800
CY121	J	830	+	60 days	1090	n.d.	n.d.
CY124	A	1510	+	59 days	1030	+	1/25 000
CY126	J	780	+	90 days	1200	+	1/64 000
CY137	J	240	n.d.	32 days	620	+	n.d.
CY140	J	520	–	24 days	390	+/–	

similar distribution (data not shown). Therefore, it is arguable that the mortality was not due to an atypical RHDV viral strain, but rather to a classical RHDV strain, a point which was also confirmed by a test of 16 liver homogenates by a differential sandwich ELISA that enabled discrimination between the classical RHDV strains and "RHDVa".

4. DISCUSSION

The data we recorded during this outbreak of RHD were unusual. Despite being seropositive for anti-RHDV antibodies, and presumably protected, some rabbits died from RHD. These results did not agree with those recorded to date, because the antibodies produced in response to all isolated RHDV virulent strains are known to protect against RHD [9, 11, 34]. Moreover, the only described non-pathogenic strain, RCV, induces antibodies that protect rabbits when challenged with RHDV [7].

To unravel this problem, we first checked by phylogenetic analysis that the viral strain was probably not a variant that could not be neutralised by classical antibodies. Then, we determined that the antibodies we detected did not result from the virus that killed the rabbits. Indeed, if the rabbits had been affected by a chronic form of RHD, then the time elapsed between the infection and their death would have been sufficient to detect at least some antibody isotypes (IgM, IgA and/or IgG) produced in response to the virus responsible for this mortality [1]. Because the rabbits had developed a classical acute disease and the viral strains involved did not differ from the other strains isolated in France in this period, we concluded that these rabbits died from RHD despite the fact that they already had antibodies when they became infected. In other words, the detected antibodies were not protective.

The origin of these antibodies remains unknown because we did not isolate the virus that induced them. Since all dead rabbits were more than two months old, it is likely that detected antibodies were not of maternal origin [13]. Furthermore, because antibodies detected by the VP60-ELISA are known to appear 5–6 days after infection [2, 16] yet RHD generally kills the rabbits 2–3 days after infection, it seems most likely that these antibodies were present before the rabbit's exposure to the virus that killed them, even though such antibodies would normally be expected to offer the rabbits some protection against the development of lethal RHD [2, 16]. Similar results have been obtained in Australia where a RHDV-like virus was shown to be present before the introduction of RHDV [32]. This study suggests that the putative Australian RHDV-like virus is different from RCV because it induces

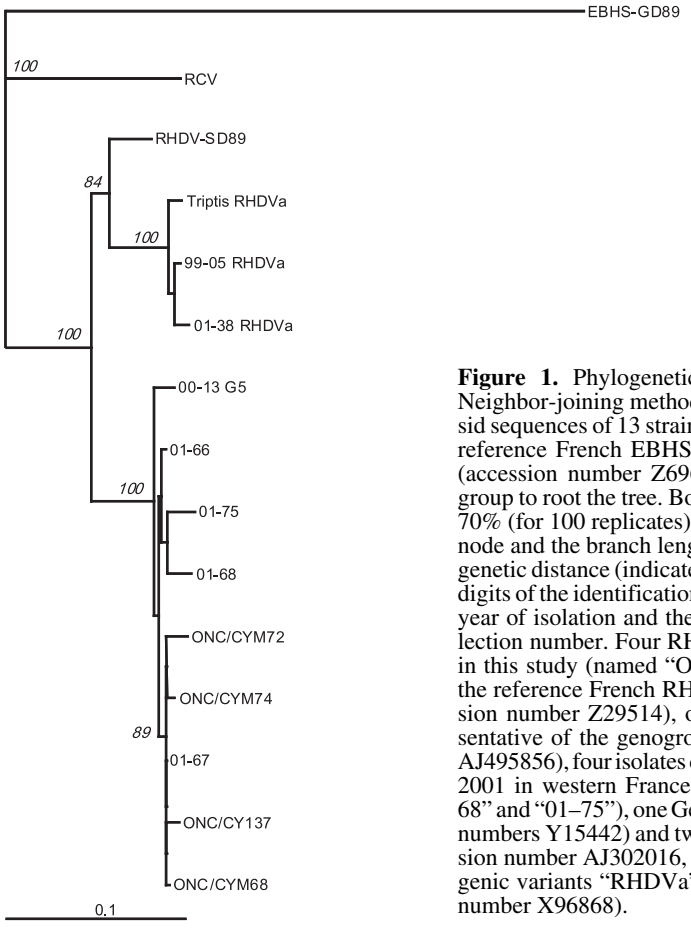

Figure 1. Phylogenetic tree derived using the Neighbor-joining method and using the partial capsid sequences of 13 strains of RHDV and RCV. The reference French EBHSV strain "EBHSV-GD89" (accession number Z69620) was used as an outgroup to root the tree. Bootstrap values greater than 70% (for 100 replicates) are given in italics at each node and the branch lengths are proportional to the genetic distance (indicated by the bar). The first two digits of the identification number correspond to the year of isolation and the last two digits to the collection number. Four RHDV isolates characterised in this study (named "ONC/") were analysed with the reference French RHDV strain "SD89" (accession number Z29514), one isolate "00–13" representative of the genogroup G5 (accession number AJ495856), four isolates collected during the autumn 2001 in western France ("01–66", "01–67", "01–68" and "01–75"), one German ("Triptis", accession numbers Y15442) and two French ("99–05", accession number AJ302016, and "01–38") RHDV antigenic variants "RHDVa", and the RCV (accession number X96868).

antibodies that do not protect against RHD with titres ≤ 1/320 [32] whereas RHDV antibodies are known to confer protection at titre = 1/20 [16]. In our study, some rabbits died with titres of 1/25 000 and 1/64 000, suggesting that the RHDV-like strain present in this study induces antibodies that do not protect against classical RHD and therefore may be different from the Australian one.

A study on the molecular epidemiology of RHDV carried out in Britain suggests a greater variability in RHDV strains than previously assumed [25]. The comparison of a portion of the capsid sequence of avirulent RHDV strains revealed no significant differences with the homologous region of the sequence of virulent RHDV strains. Moreover, the avirulent RHDV strains are more closely related to virulent RHDV than to RCV. The evidence for non-protective strains is a new development consistent with this large variability. It is therefore important to isolate and characterise these strains, and to validate immunological tests to improve the classification of antibodies induced by these different viral strains. Some authors have already proposed criteria to differentiate antibodies due to RHDV from antibodies due to RHDV-like strains [13]. These suggested criteria, based on similarities in the profiles of antibody patterns in individual rabbits, need to take into account the differences

in protective characteristics of RHDV-like strains. To date, and despite several attempts, it has not been possible to isolate such an RHDV-like virus from a wild population. The absence of clinical signs of the disease is likely responsible for these failures because there is no direct information that can be recorded when manipulating rabbits that may lead to suspect an infestation by a RHDV-like virus. For this reason, it is likely easier to detect a RHDV-like virus in domestic rabbits, as done with RCV, but it requires that no prior vaccination against RHD has been made and that a constant serological survey is carried out to detect seroconversions. Furthemore, one must try to isolate the virus during the viremia, when it is the most abundant.

The existence of a non-protective RHDV-like strain leads us to reconsider some results previously recorded. During an outbreak that occurred in 1995 in France, a high mortality was recorded despite a high proportion of rabbits carrying RHDV antibodies, revealed by VP60-RHDV ELISA tests [21]. Because some cases of myxomatosis were detected during the outbreak of RHD, the authors assumed that the immunosuppressive characteristics of the myxoma virus could have increased vulnerability to RHD. Our results lead us to add a new hypothesis: the detected antibodies may have been non-protective, which may explain this high rate of mortality in a population that seemed to be protected.

The potential role of non-pathogenic strains of RHDV-like viruses in RHDV epidemiology is currently under investigation, with a key question being whether these strains might protect against RHD [12, 37]. The existence of non-protective strains and their competition with protective strains is a new factor that should also be taken into account in these studies, because it could counterbalance the benefit of the protective strains given to the rabbits. Serological surveys should use tests that are able to distinguish between antibodies that are protective against RHD and antibodies that are not. Additionally, a programme aimed at isolating the different RHDV-like strains should be a priority. Also, their pathogenicity and the level of protection conferred by their antibodies should be determined in order to better understand the epidemiology of RHD in wild populations.

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