

AN UPDATE ON THE PRESENCE AND SPREADING IN ITALY OF RABBIT HAEMORRHAGIC DISEASE VIRUS AND OF ITS ANTIGENIC VARIANT RHDVa

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ABSTRACT

Rabbit haemorrhagic disease virus (RHDV) is a non-cultivable calicivirus that infects rabbits (*Oryctolagus cuniculus*) and causes an acute fatal hepatitis, firstly described in China in 1984. The first consistent antigenic variant originated from the classical strain, called RHDVa, was identified almost contemporaneously in Italy and Germany in 1997. We report here the results of diagnostic studies in order to compare the rate of diffusion of RHDV and RHDVa in Italy since its first detection. From 2000 to 2003 RHDVa has been identified in 53,7% of the cases and, in particular during the last two years, a total of 201 cases of RHD were diagnosed, 128 (63,7%) of which resulted RHDVa. The higher percentages (70-100%) were found in those regions where is concentrated most of intensive rabbit production (Lombardia, Emilia Romagna, Piemonte, Veneto and Campania). This survey shows that RHDVa is present in most part of Italy and that it is rapidly replacing the RHDV “classical” strain. The importance of such variant is discussed with reference to vaccine preparation and application.

Key words: RHDV, antigenic variants, diagnosis, Italy.

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of the European rabbit (*Oryctolagus cuniculus*), caused by a calicivirus (RHDV). (OHLINGER

1990; CAPUCCI, 1991). RHD was first reported in 1984 in China (LIU *et al.*, 1984), coming out in Europe two years later where it caused a succession of devastating epidemics both in commercial and wild rabbits. Currently RHD is endemic in East Asia, Europe, Australia and New Zealand. Outbreaks have also been recorded in Central America (Mexico and Cuba), Saudi Arabia and West and North Africa. In 2000 and 2001 three independent outbreaks were recorded in the USA. All known RHD viral isolates appear to belong to one serotype and the molecular epidemiological studies, carried out sequencing the gene of the RHDV capsid protein (VP60) of different isolates, did not detect consistent antigenic changes in amino acid composition (differences between 2% and 5%) up to the end of 1996 (LE GALL 1998; NOWOTNY 1997). Nevertheless, isolates that exhibit temperature-dependant differences in haemoagglutinating characteristics (CAPUCCI *et al.* 1996) have been described, and more recently a consistent genetic and antigenic RHDV variant was simultaneously identified in Italy (CAPUCCI *et al.*, 1998) and Germany (SCHIRMAIER *et al.*, 1999). This variant, named RHDVa, has been considered as a subtype of the original virus and therefore specific ELISA tests based on newly developed MAbs towards RHDVa have been set up with the aims to rapidly distinguish between outbreaks caused by RHDV and RHDVa and to enlarge the capacity to detect new possible variants. The rate of diffusion of RHDV and RHDVa in the field during the period 1997-1999 has been previously presented (GRAZIOLI *et al.* 2000); now we report the results of diagnostic studies in order to compare the rate of diffusion of RHDV and RHDVa in Italian regions during the last four years (2000-2003).

MATERIAL AND METHODS

Sampling

In the study we included both the RHD positive samples (liver and spleen homogenates) directly conferred to the IZSLER of Brescia for virological diagnosis, and all the RHDV positive samples, sent to us from diagnostic laboratories of other regions involved in the study, in order to mark them as RHDV or RHDVa. The total number of samples considered in the present work and their origin, divided for each year, are reported in table 1 and 2. Thanks to the new electronic program for registering samples and results (DarWin), implemented at our institute on 2002, it is also possible for the last two years (2002-2003) to exactly indicate the number of RHD outbreaks and the rate of RHD positive over the total number of rabbits examined.

Virus detection (sandwich ELISA)

The organ homogenates (liver and spleen if available), from one or more rabbits from each outbreak, were examined using the differential sandwich ELISA test. This method (CAPUCCI *et al.* 1995; CAPUCCI and LAVAZZA 2004) is based on the use at the same time of a polyclonal serum and of a panel of MAbs, and it has been recently improved with the addition of antibodies produced towards the RHDVa subtype (CAPUCCI, *et al.* 1998; GRAZIOLI *et al.* 2000). The hyperimmune serum is used as tracer in order to have the broadest spectrum of reactivity towards RHDV-like strains, thus giving to the method a

high level of sensitivity. The panel is constituted of three groups of MAbs: i) MAbs specific for epitopes expressed by the original “classical” RHDV strain (BS89). ii) MAbs reacting both with RHDV and RHDVa which recognise internal epitopes, normally buried inside the virus but exposed after viral degradation, useful to detect virions present during chronic evolutions of the disease. iii) MAbs specific for RHDVa.

The technical procedure and the steps for performing such method are already described in details elsewhere (CAPUCCI and LAVAZZA 2004).

Table 1. Summary of the number and percentage of RHDV and RHDVa subtype isolates from 1997 to 1999 (modified from GRAZIOLI et al 2000)

Region	Year Type of isolate	1997		1998		1999		Total					
		RHDV (%)	RHDVa (%)	RHDV (%)	RHDVa (%)	RHDV (%)	RHDVa (%)	RHDV (%)	RHDVa (%)				
North	Piemonte	-	-	-	-	6	46,1	7	53,9	6	46,1	7	53,9
	Triveneto	20	90,1	2	9,9	2	33,3	4	66,7	3	33,3	6	66,6
	Lombardia	68	97,2	2	2,8	38	92,7	3	7,3	30	69,7	13	30,3
	Emilia Romagna												
Centre	Lazio & Toscana	42	72,4	16	27,6	17	70,8	7	29,1	-	-	-	-
	Marche	-	-	-	-	-	-	-	-	-	-	-	-
	Abruzzo	-	-	-	-	-	-	-	-	-	-	-	-
South	Campania	-	-	-	-	-	-	-	-	-	-	-	-
	Puglia	-	-	-	-	-	-	-	-	-	-	-	-
	Sicilia	61	100	0	0,0	9	100	0	0,0	-	-	-	-
Total pos.		191	90,5	20	9,5	66	82,5	14	17,5	39	60,0	26	40,0
						296	83,1	60	16,9				

RESULTS AND DISCUSSION

As previously reported (Table 1) RHDVa was already present in some part of Italy in 1997 i.e. at the time of its first identification, and in the following years it showed the tendency to increase its spreading quite rapidly on the field and especially in those regions where intensive rabbit production is more practised (es. 3% and 9% of RHDVa isolates in 1997 vs 30% and 66% of the RHD cases in 1999 respectively in Lombardia & Emilia Romagna and Triveneto). By looking at Table 2 and at Figure 1 the tendency of RHDVa to progressively substitute the original “classic” RHDV strain is much more evident. Again, it looks to be highly predominant in those regions where industrial rabbitries are more concentrated either in North (Piemonte, Lombardia, Emilia Romagna) or South Italy (Campania). From our data it looks to be widespread present all over Italy with the exception of Sicilia from where all the examined samples, including also some wild rabbits, belonged to the original RHDV.

Table 2. Summary of the number of RHDV and RHDVa subtype isolates diagnosed from 2000 to 2003

Region	Year	2000		2001		2002		2003		Total	
	Type of isolate	RHD V	RHD Va	RHD V	RHD Va	RHD V	RHD Va	RHD V	RHD Va	RHD V	RHD Va
North	Piemonte	7	4	-	-	0	1	0	7	7	12
	Triveneto	1	6	3	7	-	-	7	1	11	14
	Lombardia			10	20	11	39	0	17		
	Emilia Romagna	81	35	12	5	29	34	4	11	147	161
	Romagna										
Centre	Lazio & Toscana	-	-	-	-	-	-	-	-	-	-
	Marche	-	-	-	-	-	-	1	0	1	0
	Abruzzo	-	-	0	5	-	-	-	-	0	5
South	Campania	-	-	0	7	0	3	0	15	0	25
	Puglia	-	-	-	-	1	0	-	-	1	0
	Sicilia	-	-	-	-	20	0	-	-	20	0
Total pos.		89	45	25	44	61	77	12	51	187	217

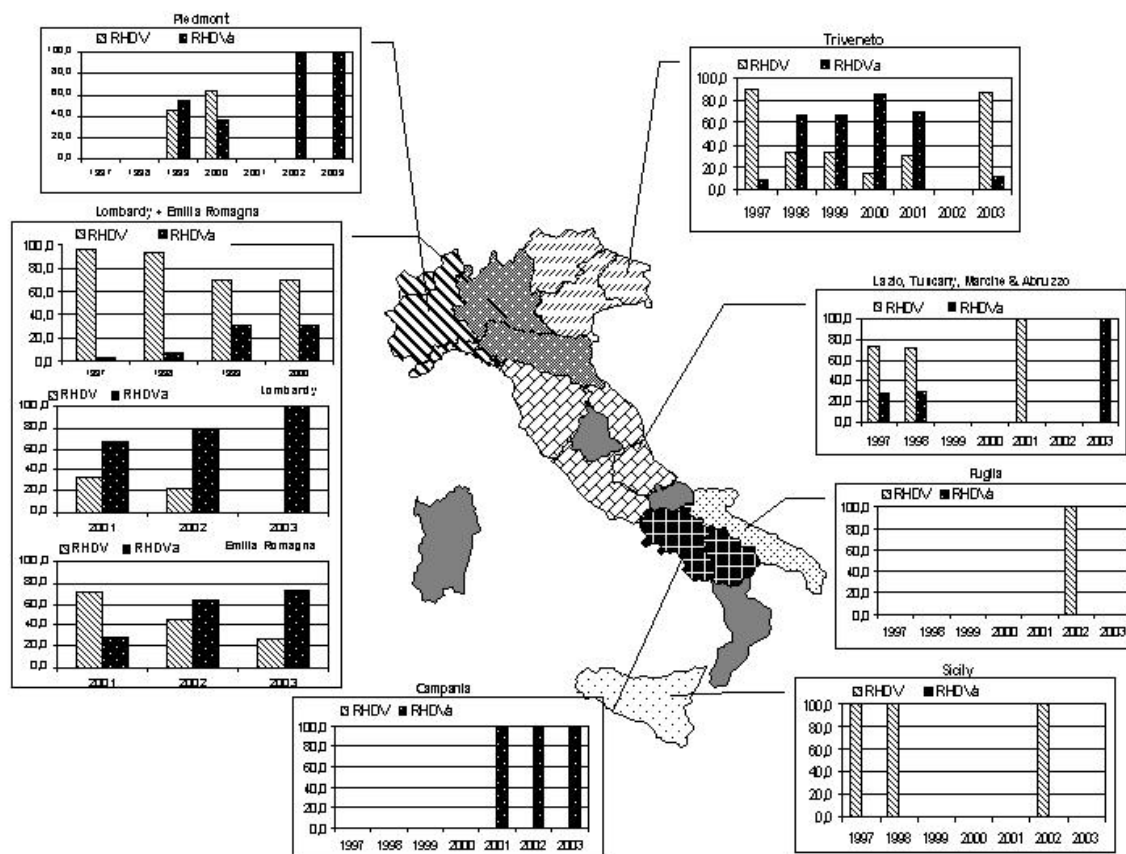


Figure 1. Graphical representation of RHDV and RHDVa isolates in the different Italian regions expressed as percentage of RHD positive samples

Table 3. Positive outbreaks of RHD diagnosed in Italy from 1999 to 2003

Year	Tot. examined	Total RHD pos. (%)	RHDV pos. (%)	RHDVa pos. (%)
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%)

Regarding the diffusion of RHDVa outside Italy it is known that the variant has been identified almost contemporaneously in German (Schirraier et al 1999) but it also caused the first outbreaks of RHD in Iowa (USA) on spring 2000 and it has been detected in France (CAPUCCI personal observations). In the last two years rabbits' liver samples were sent from France (wild rabbits), Germany and Belgium (commercial rabbits) to the OIE Reference Laboratory for typing the RHDV strain: only in the case of the German outbreak we were able to detect RHDVa (data not shown), whilst the Belgian and France isolates showed the MABs profile typical of the classical strain.

From a more general point of view, the diagnostic data obtained in the last 10 years seem to indicate in Italy a stable incidence of RHD in the time and a modest decrease just in 2003 (Table 3). If the number of outbreaks of the disease could have been underestimated in the '90, considering that most of the cases occurred in rural units, which owners learnt to recognise the disease and did not send samples to the diagnostic laboratory, different is the situation nowadays. In fact, during the period 2002-2003 a considerable number of outbreaks were observed in industrial farms where vaccination was normally practised in breeding units. In such outbreaks RHD has appeared with reduced levels of morbidity and mortality mainly in meat animals, which are itself naturally resistant till the age of 45-50dd, but it sporadically killed also some does thus making more difficult to put forward a clinical diagnosis of RHD and suggesting to confirm always the suspect by laboratory examinations.

Most people have commonly interpreted this situation as consequence of the "breakdown of immunity" induced by vaccination. Indeed, it is important to note that rabbits vaccinated with classical RHDV strains (the only commercially available by now) and then challenged with RHDVa variant do not show any sign of disease, even if rabbits vaccinated with a low dose are less resistant to the variant than to the original strain of RHDV used as vaccine (CAPUCCI and LAVAZZA, personal observations). Nevertheless, it is admitted that in field conditions it is easier to achieve a lower level of vaccinal protection due to the fact that the global conditions of vaccination are completely different from those experimentally applied in laboratory. In fact, in industrial rabbitries either the administration procedures and the vaccination schemes are rarely equal or even similar to those practised in laboratory conditions, and the environmental and management conditions are often so bad to determine stress factors able to limit the capacity of animals to correctly develop an immunity response. Anyway, we should also consider that among the causes that could have contributed to the selection of the RHDVa variant there is the selective pressure on the virus applied by regularly vaccinated rabbits. Therefore, the genomic and antigenic mutation that has occurred on

the structure of RHDV and exactly on its main neutralizing epitope (CAPUCCI et al 1995; CAPUCCI et al 1998), which resulted in the appearance of the new variant RHDVa, can be considered as a strategic attempt of the virus for surviving.

CONCLUSIONS

Our diagnostic surveys demonstrate that the subtype RHDVa has appeared in Italy in 1997 and it has from then on diffused quite rapidly so that it first reached level comparable to the original RHDV and then it passed over resulting in the more frequently RHDV strain identified in those Italian regions where rabbit production is more developed (Lombardia, Piemonte, Emilia Romagna, Veneto and Campania). In the last year around 80% of the outbreaks was caused by RHDVa. Considering the consistent antigenic difference between RHDV and RHDVa, it should be strongly recommended to continue to follow the evolution of RHD on the field, in particular for what concerns the capacity to protect rabbits against infection with RHDVa by immunization with standard vaccines, in order to be ready to use in the future the best antigen to prepare the vaccines if there will be evidence of reduction or lack of protection.

In conclusion, we can affirm the quasispecies nature of this RNA virus involved in the aetiology of RHD and we stress the importance of a continuous epidemiological and antigenic surveillance. Indeed, the results show that virologic methods of diagnosis, often in combination of serological methods, provides novel and highly sensitive means for the identification and characterisation of such viruses, with special regard to genome composition, evolution, features of pathogenicity and molecular epizootology.

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