RESULTS OF SEROEPIDEMIOLOGICAL SURVEYS FOR THE DETECTION OF NATURAL ANTI-RHD ANTIBODIES INDUCED BY THE NONPATHOGENIC RABBIT CALICIVIRUS (RCV) IN MEAT RABBITS

CAPUCCI L.[1,2], CERRONE A.[3], BOTTI G.[1,2], MARIANI F.[3], BARTOLI M. [3], LAVAZZA A.[1,2]

[1]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and
[2]OIE Reference Centre for Haemorrhagic Disease of Lagomorphs,
Via A. Bianchi 9, 25124 Brescia (Italy),
alavazza@bs.izs.it , capucci@bs.izs.it
[3]Istituto Zooprofilattico Sperimentale del Mezzogiorno,
Via della Salute 2, 80055 Portici (NA) (Italy),
annacerr@tin.it

ABSTRACT

Rabbit haemorrhagic disease virus (RHDV) is a non-cultivable calicivirus that infects rabbits *(Oryctolagus cuniculus*) and causes outbreaks of an acute fatal hepatitis, firstly described in China in 1984. Another virus, named rabbit calicivirus (RCV), related to the RHDV was identified in healthy rabbits in Italy in 1996. This virus is avirulent, replicates in the intestine at a low titre and presents a 92% genomic identity with RHDV. In addition, seroepidemiological data have shown the presence of 'naturally acquired' antibodies in Europe either from 1975 to 1987 i.e. before the first evidence of the disease and in colonies where RHD had never been recorded or vaccination performed, thus suggesting the existence of one or more non-pathogenic viral strain antigenically related to RHDV. In order to check the diffusion of RCV in Italian rabbitries we conducted a survey respectively in 39 farms in North Italy (Lombardia and Triveneto) and 21 farms in South Italy (Lazio, Campania and Basilicata) by testing non vaccinated 80 dd old meat rabbits at slaughterhouse. The results indicate the presence of “natural antibodies” presumably induced by RCV i.e. over 80% of animals showing titres ≥ 1/20 in almost 30% of farms controlled.

Key words: RHDV, RCV, seroepidemiological surveys, 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). Another virus, named rabbit calicivirus (RCV), related to the RHDV was identified in healthy rabbits. It is significantly different from the previously characterised RHDV isolates in terms of pathogenicity, viral titer, tissue tropism, and primary sequence of the structural protein i.e. it is avirulent, replicates in the intestine at a low titre and presents a genomic identity with RHDV of around 92% (CAPUCCI *et al* 1996; CAPUCCI *et al* 1997). Indeed, seroepidemiological data suggest the existence of

one or more 'non-pathogenic' viral strain antigenically related to RHDV. In fact, the presence of so-called 'naturally acquired' antibodies was shown in Europe either from 1975 to 1987 i.e. before the first evidence of the disease (RODAK *et al*. 1990) and in colonies where RHD had never been recorded or vaccination performed (TROUT *et al*., 1997; MARCHANDEAU *et al*. 1998). The existence of non-pathogenic caliciviruses provides a likely explanation for the early discrepancies found in the course of serological surveys of the rabbit populations in European countries as well as in Australia and New Zealand (COOKE *et al*. 2002; ROBINSON *et al*. 2002). Since the antibodies against RCV and the putative non-pathogenic RHDV–like viruses could confer a variable level of protection against RHD, the possible role of these non-pathogenic RHDV-like viruses in reducing the impact of RHD has been suggested (COOKE *et al*. 2002).
In order to check the diffusion of RCV in Italian rabbitries we conducted a two separate serological surveys: the first during 1999 in 39 farms located in North Italy (Lombardia and Veneto) and the second during 2002-2003 in 21 farms located in Central (Lazio) and South Italy (Campania & Basilicata) by testing, for the presence of anti-RHD antibodies, non vaccinated 80dd old meat rabbits at slaughterhouse.

MATERIAL AND METHODS

Antibodies detection

The serological test used in routine is based on a competitive ELISA (cELISA), which was standardized using sera from commercial rabbits, i.e. the main target of the assay. In the first phase of standardization it was immediately evident the presence of sera classify as positive (clearly above the cut-off value), even if belonging to apparently uninfected, healthy populations. Since these sera were strongly related to and repeatedly found in particular units, the existence of a non-pathogenic calicivirus highly related to RHDV was initially suggested and then demonstrated (CAPUCCI *et al* 1996; CAPUCCI *et al* 1997). Therefore, the cELISA, initially developed for using in the RHD serology, really detects also the antibodies induced by the non-pathogenic virus. Considering the high correlation between the two viruses, it is possible to predict a RHDV infection by inference from the serological results alone, only if the cELISA titres reach high values (>1/1280) typical of RHDV convalescent rabbit and never detected in RCV infected ones. The technical procedure and the steps for performing such method are already described in details elsewhere (CAPUCCI and LAVAZZA 2004). Moreover, in order to improve the serological interpretation and for correctly classifying the immunological status of rabbits, two different anti-isotype ELISA techniques were employed in order to detect specific IgA and IgM anti-RHD antibodies (Cooke *et al*, 2000). The technical procedure and the steps for performing such method are already described in details elsewhere (CAPUCCI and LAVAZZA 2004).

Sampling

The seroepidemiological survey was carried out in two separate periods and geographical areas. In both cases, however, different groups 78-82 dd old rabbits were sampled and a set of at least 35 sera were taken at slaughterhouse from each group.

Table 1. Details of the sera sampled during the second phase of the survey with indication of the results obtained for each group.

N. Farm	N. Prot.	N. Slaughter house	Date sampling	Tot sera examined	Tot neg	% neg	Tot pos	% pos	Result
1	12av	2	08/07/2002	40	40	100,0	0	0,0	N/P
	17av	2	13/01/2003	40	9	22,5	31	77,5	
2	19bn	3	25/06/2002	40	1	2,5	39	97,5	P
3	30ce	4	09/09/2002	36	6	16,7	30	83,3	P
	31ce	4	30/09/2002	40	2	5,0	38	95,0	
	36ce	4	27/01/2003	33	13	39,4	20	60,6	
4	22bn	3	05/11/2002	40	38	95,0	2	5,0	N
	24bn	3	26/11/2002	40	40	100,0	0	0,0	
	27bn	3	11/02/2003	40	40	100,0	0	0,0	
5	1sa	1	03/07/2002	40	0	0,0	40	100,0	P
	6sa	1	05/11/2002	40	1	2,5	39	97,5	
	8sa	1	10/12/2002	40	2	5,0	38	95,0	
6	29ce	4	17/07/2002	40	40	100,0	0	0,0	N
	33ce	4	04/11/2002	40	40	100,0	0	0,0	
	34ce	4	25/11/2002	40	40	100,0	1	2,5	
7	45na	5	20/02/2003	42	42	100,0	0	0,0	N
	46na	5	27/02/2003	42	42	100,0	0	0,0	
8	20bn	3	23/07/2002	40	15	37,5	25	62,5	D
	21bn	3	24/09/2002	40	20	50,0	20	50,0	
9	13av	2	17/07/2002	40	40	100,0	0	0,0	N
	15av	2	18/09/2002	40	40	100,0	0	0,0	
10	39na	5	23/10/2002	40	21	52,5	19	47,5	D
	40na	5	20/11/2002	40	30	75,0	10	25,0	
	41na	5	28/11/2002	38	38	100,0	0	0,0	
11	38na	5	10/07/2002	35	28	80,0	7	20,0	D
	44na	5	14/02/2003	40	27	67,5	13	32,5	
12	43na	5	07/02/2003	40	18	45,0	22	55,0	D
13	23bn	3	19/11/2002	40	39	97,5	1	2,5	N
	25bn	3	10/12/2002	40	40	100,0	0	0,0	
14	3sa	1	04/09/2002	40	40	100,0	0	0,0	N
	4sa	1	18/09/2002	41	41	100,0	0	0,0	
	7sa	1	20/11/2002	40	40	100,0	0	0,0	
	9sa	1	08/01/2003	40	40	100,0	0	0,0	
15	42na	5	05/12/2002	40	40	100,0	0	0,0	N
16	26bn	3	08/01/2003	40	2	5,0	38	95,0	P
17	28ce	4	01/07/2002	40	40	100,0	0	0,0	N
	35ce	4	09/12/2002	40	40	100,0	0	0,0	
18	2sa	1	10/07/2002	40	40	100,0	0	0,0	N
	5sa	1	07/10/2002	39	38	97,4	1	2,6	
	10sa	1	28/01/2003	40	40	100,0	0	0,0	
19	37ce	4	10/03/2003	40	25	62,5	15	37,5	D
20	32ce	4	16/10/2002	40	40	100,0	0	0,0	N
21	11av	2	01/07/2002	40	40	100,0	0	0,0	N
	14av	2	11/09/2002	40	40	100,0	0	0,0	
	16av	2	13/11/2002	40	40	100,0	0	0,0	

The first survey was conducted during 1999 in one big slaughterhouse located in Lombardia region where are usually processed rabbits originating from farms located in Lombardia and Triveneto (Veneto, Friuli-Venezia-Giulia and Trentino-Alto-Adige). In total we took 39 different groups of rabbit sera from an equal number of farms.

The second survey was conducted between June 2002 and March 2003 in 5 different slaughterhouses located in Campania (South Italy) where are usually processed animals rabbits originating from either intensive or semi-intensive farms located in Campania, Basilicata and Lazio. A total of 45 sets of sera where take from 21 different farms; thus each farm was controlled at least once and maximum four times (Table 1).
In the sampling procedures great attention was put in order to choose homogeneous group of animals i.e. rabbits coming from the same unit and possibly the same shed, and to record for each group a detailed history and a full description of the farm of origin. During the second phase of the survey it was also possible to take multiple sets of sera from the same farm at different time-points along a six months period. Indeed, we decided to include also some mixed groups of animals originating from small semi-intensive units distributed in a rural area of Lazio region.

RESULTS AND DISCUSSION

The results, obtained during the first period of the survey in North Italy, are reported in Table 2. In 24 groups of rabbits, each group being composed by at least 35 rabbits and corresponding to a single rabbit farm, almost all the rabbits tested (>95%), resulted negative for anti-RHDV antibodies, as expected since meat rabbit are usually not vaccinated and maternal antibodies are normally detected till the age of 45dd maximum (LAVAZZA & CAPUCCI, personal observation). On the contrary more than 60% of the animals of 13 groups resulted seropositive with titres between 1/20 and 1/320. These titres must be considered low/medium in value and similar to those induced by vaccination. Nevertheless anamnestic data proved that no RHD outbreaks have occurred in the farms of origin since more than one year, neither the meat rabbits had been vaccinated for RHD. Finally, in 2 groups the percentage of seropositive animals was around 5-10% thus making impossible a reliable explanation on the nature of the serological titres.

The results, obtained during the second period of the survey in Central and South Italy, are reported in Table 1 and 2. In total, the positive sera were 516 out of 1786 examined (28.9%) and as in the first survey the titres detected were mostly low or medium (1/20-1/320) being only seven the sera with titres 1/640-1/1280. In two of the four positive farms (n. 3 and n. 5) the serological positivity was confirmed by three consecutive samples along a 5 months period. Similarly in all the negative farms with the exception of one (n. 20), the serological negativity was confirmed by two to four controls done within a variable period of time (1-5 months). In one farm (n. 1) 6 months after the first control resulted completely negative, 77,5% of the rabbits resulted positive at the second sampling. In the last five farms (n. 8, 10, 11, 12, 19) the rate of positive rabbits was ranging between 20 and 60%. It is difficult to fully explain the reasons of such partial seroprevalence, but it could be partially due to the fact that two (n. 10 and 11) were

mixed groups of animals originating from several small semi-intensive units distributed in a rural area of Lazio region and another group (n.8) was originating from a farms composed by multiple and independent sheds for meat rabbits. No reasonable explanations there are for the last two groups (n. 12, 19).

Table 2. Results of the serological surveys

Serological results	Criteria applied	N. groups (%)			
Positive	> 75% of rabbits positive	13	(33.3%)	4	(19.1%)
Doubtful	5-10% of rabbits positive	2	(5.2%)	0	(0,0%)
	20-60% of rabbits positive	0	(0,0%)	5	(23.8%)
Negative to positive	from 0% to >75% of rabbits positive	0	(0,0%)	1	(4.7%)
Negative	> 95% of rabbits negative	24	(61.5%)	11	(52.4%)
	Total	39 Lombardy		21 Campania	

The results obtained using isotype ELISAs (Table 3) and particularly the presence of IgA in all the tested samples, sometimes at very high titres, clearly indicate that the antibodies found at slaughtering were the results of an active immunization with an infectious agent and not maternal antibodies, that are usually IgG, or induced by vaccination since in this case IgA are not detected. It is indeed notable that IgM were detected (farms n. 5 and 16) only in association with lower, but presumably increasing, titres of IgA (CAPUCCI *et al* 1991, COOKE *et al*. 2002).

Table 3: Results of isotype ELISAs for detecting anti-RHDV IgA and IgM

N. Farm	N. Prot.	Date sampling	Tot sera examined	IgA pos (%)	IgA titre (1/..) in percentage					IgM pos (%)
					160	640	2560	10240	≥1024(	
1	17av	13/01/2003	9	100	0.0	11.1	55.6	22.2	11.1	0.0
3	31ce	30/09/2002	11	100	0.0	36.4	36.4	9.1	18.1	0.0
5	6sa	05/11/2002	16	100	18.7	37.5	43.8	0.0	0.0	37.5
16	26bn	08/01/2003	10	100	0.0	60.0	20.0	20.0	0.0	10.0

CONCLUSIONS

The results of serological surveys conducted with the aim to investigate inside different commercial rabbit groups originating from different areas of Italy for the presence of the non-pathogenic virus correlated to RHDV, clearly show that antibodies reactive with RHDV are present in some rabbit populations. The main question that arises from the finding of RHDV cross-reactive antibodies is whether these antibodies could interfere with the RHDV infection and, therefore, with the course of the disease. The data obtained in this study suggest that could be such case. In fact the test here used - the

competition ELISA considered the standard and reference test for RHD of our laboratory - mainly measures antibodies directed against antigenic determinants on the external surface of the virus, usually the most specific and functionally important. Experimental data (CAPUCCI and LAVAZZA pers. observations) showed that there is a firm correlation between the titre in cELISA and the state of protection from the disease i.e. rabbits with titres equal or higher than 1/10 for antibodies specifically induced by RHDV did not show any sign of disease when challenged with virulent RHDV.

Since no viral identifications were attempted, we have no direct proofs of the presence and circulation of a virus referable to RCV in the farms of origin of the controlled rabbits resulted positive. Nevertheless we are confident that the anti-RHDV antibodies found were the results of an active infection with an RCV-like virus. This for at least three reasons: 1) anti-isotype ELISAs clearly demonstrated the presence of IgA and thus of an active infection 2); there was no evidence of overt RHD clinical disease from the history of the rabbitries; 3) it is also known that such type of reactivity using cELISA makes RCV different from other RHDV-like strains, which existence in Europe, Australia and New Zealand has been only inferred by serological data. In these cases the comparison of the results obtained using different ELISAs systems (sandwich Indirect ELISA, solid phase ELISA, cELISA with degraded antigen) providing different level of specificity, indicated that the great part of the antibodies detected were reacting with antigenic determinants buried inside the structure of the RHDV capsid. Since these epitopes could be considered as “common” (and possibly “group specific”) in all the calicivirus of lagomorphs, the serological pictures achieved suggested the presence of infectious agents correlated with RHDV, but different from RCV.

In conclusion, the results show that the use of serological methods of diagnosis provides novel and highly sensitive and specific means for the identification and characterisation of novel viruses inside the group of calicivirus of lagomorphs and may help to better understand the pathogenesis of the infection that they can cause.

ACKNOWLEDGEMENTS

We would like to thank M. Cerioli, C. Palotta and G. Bozzoni for Technical Assistance. This study has been financed by Ministero della Salute – Current Research 2003

REFERENCES

CAPUCCI L., FUSI P., LAVAZZA A., PACCIARINI M.L., ROSSI C. 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. *J. Virol.,* **70**:8614–8623.

CAPUCCI L. & LAVAZZA A. (2004) Rabbit haemorrhagic disease, in: *Manual of standards for diagnostic tests and vaccines*. Fifth edition, OIE Edition, Paris, 2004, pp. In press.

CAPUCCI L., NARDIN A., LAVAZZA A. 1997. Seroconversion in an industrial unit of rabbits infected with a non-pathogenic rabbit haemorrhagic disease-like virus. *Vet. Rec.*, **140**:647–650.

CAPUCCI, L., SCICLUNA, M.T., LAVAZZA, A., 1991. Diagnosis of viral hemorrhagic disease of rabbits and European brown hare syndrome. *Rev. sci. tech. Off. int. Epiz*. **10**:347-370.

COOKE B.D., MCPHEE S., ROBINSON A.J., CAPUCCI L. 2002. Rabbit haemorrhagic disease: does a pre-existing RHDV-like virus reduce the effectiveness of RHD as a biological control in Australia?, *Wildl. Res*. **29**:673-682.

MARCHANDEAU S., RICCI J.-C., CHANTAL J. 1998. Taux de prévalence sérologique du virus de la maladie virale hémorragique (VHD) du lapin de garenne (Oryctolagus cuniculus) et de ses formes apparentées au sein de différentes populations sauvages en France, *Mammalia* **62**:95-103.

OHLINGER V.F., HAAS B., MEYERS G., WEILAND F., THIEL H.J., 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. *J. Virol.* **64**:3331-3336.

ROBINSON A.J., KIRKLAND P.D., FORRESTER R.I., CAPUCCI L., COOKE B.D., PHILBEY A.W. 2002. Serological evidence for the presence of a calicivirus in Australian wild rabbits, Oryctolagus cuniculus, before the introduction of rabbit haemorrhagic disease virus (RHDV): its potential influence on the specificity of a competitive ELISA for RHDV, *Wildl. Res*. **29**:655-662.

RODAK L., SMID B., VALICEK L., VESELY T., STEPANEK J., HAMPL J., JURAK E. 1990. Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins, *J. Gen. Virol*. **71**:1075-1080.

TROUT R.C., CHASEY D., SHARP G. 1997. Seroepidemiology of rabbit haemorrhagic disease (RHD) in wild rabbits (Oryctolagus cuniculus) in the United Kingdom, *J. Zool. London* **243**: 846-853.