ROTAVIRUS IN DIARRHEIC RABBITS: PREVALENCE AND CHARACTERIZATION OF STRAINS IN ITALIAN FARMS

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ABSTRACT

The multifactorial rabbit enteropathy has a great importance in rabbit meat production for its economical impact. Stating the pathogenic role and importance as primary agent of rotavirus, the purposes was to estimate the prevalence of lapine rotavirus (LRV), identified by negative staining electron microscopy (nsEM), on samples from rabbits showing either a "generic" enteropathy or lesions referable to mucoid enteropathy-caecal impaction and then to relate their presence with the symptoms and lesions observed. During the 2002-2007, 243 samples taken mainly from rabbits showing catarrhal, haemorrhagic or necrotic entero-tiflitis, mucoid enteropathy and caecal impaction were examined. By nsEM, the presence of viral particles was observed in 45.3% of them; rotavirus was identified in 16.0%, coronavirus-like virus in 24.7%, parvovirus in 9.0% and enterovirus-like virus in 5.8% of the positive samples. In addition, adenovirus, calicivirus and reovirus were sporadically found and, in 29 cases, 2 or 3 different viruses were contemporarily observed in association in the same sample. Using the criteria for the classification of rotavirus strains based on the VP4 (P type) and VP7 (G type) genotyping, almost all the strains were characterized as P[22] G3 confirming the presence of the newly-recognized rotavirus P[22] VP4 allele in Italian rabbits. The availability of the results of microbiological and parasitological analysis allowed to correlate nsEM observations with the contemporary presence of others pathogenic agents such as rotavirus, enteropathogenic *Escherichia coli* (EPEC) and *Staphyloccoccus aureus* in suckling rabbits; EPEC, *Cl. spiroforme* and flagellate protozoa in the others. The pathogenic role and importance of rotavirus as primary aetiological agent rabbit enteritis are discussed.

Key words: Rotavirus, Virus infection, Rabbit, Enteritis, Genotyping.

INTRODUCTION

Enteric diseases have an important role in rabbitries because they cause severe economic losses due to mortality, growth depression and worsening of conversion index. Group A rotavirus, member of the Reoviridae family, is considered the main cause of acute viral gastroenteritis in different animals including rabbits (Schoeb *et al.*, 1986). Lapin Rotavirus (LRV) is considered only mildly pathogenic (Thouless *et al.*, 1988), but it can primarily cause enteric disease in post-weaning rabbits. In addition it could also be involved in the aetiology of severe enteritis outbreaks in association with *Coli*, *Clostridium* spp, parasites and other viruses. Rabbits become infected by the oro-fecal route and the extension and the severity of the lesions (microvillus degeneration, malabsorption and diarrhoea) are dose dependent i.e. the consequences of the infection are higher when the infectious dose is also high.
The persistence of maternal antibodies until 30-45 days can reduce the symptoms of the disease. Thus till 4-5 weeks of age rabbits mostly became sub-clinical infected with particles excretion for only 3 days. The LRV infection is more frequent in 35-50 days old growing rabbits and is characterised by a high rate of morbidity and not specific clinical signs (i.e. diarrhoea, anorexia, depression). Diarrhoea appears at the beginning of viral excretion that lasts for 6-8 days, and is generally followed by

constipation. Lesions observed at necropsy are not constant: catarrhal, haemorrhagic or necrotic entero-tiflitis and caecal impaction. Ill rabbits can die due to dehydration and secondary bacterial infections whereas those that recover from the infection commonly show a decrease in productivity due to reduced absorption capacity.
Rotavirus was detected in 16.4% (Nieddu *et al*., 2000) and 23% of post-weaning rabbits with enteric signs (Cerioli *et al.,* 2004) but 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). Virological diagnosis can be achieved by testing faeces and intestinal contents by ELISA, negative staining Electron Microscopy (nsEM) and PCR. 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 neutralizing antibodies and induce a protective immunity response. Based on either antigenic or genetic characterizations, 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. Analysis of the few strains identified in various parts of the world (Canada, USA, Japan, Italy) has revealed a substantial antigenic/genetic homogeneity of LRVs, as all the viruses analyzed so far belong to the VP7 serotype G3 (Ciarlet *et al*., 1997; Conner *et al*., 1988; Petric *et al*., 1978; Sato *et al*., 1982; Thouless *et al.,* 1988) and to the VP4 serotype P11[14] (Ciarlet *et al*., 1997; Hoshino *et al*., 2002).
The aim of this study is to report the prevalence of rotavirus viral infection, in farms where outbreaks of enteritis complex were observed, in association with other virus and/or bacteria and then to report some data of genomic characterization of the LRV isolates. The tested samples were taken from rabbits showing either a “generic” enteropathy or lesions referable to mucoid enteropathy-caecal impaction and the diagnosis of enteric viruses was achieved by using negative staining electron microscopy methods.

MATERIALS AND METHODS

Animals and farms

During the period 2002-2007, we examined 243 samples taken mainly from rabbits showing catharral, haemorrhagic or necrotic entero-tiflitis and/or typical signs referred to mucoid enteropathy and caecal impaction. From each outbreak more than one animal was sampled: they were mainly meat rabbits 40-65 days old but in some cases, when clinically affected, also does and lactating rabbits were examined. In the second part of the study the virologic results were correlated with those of bacteriological and parasitological analysis performed during the period 2002-2005 on the same rabbits showing enteritis, including all visceral organs and/or skin lesions with only exception of those caused by myxomatosis. This was possible thanks to the application of the new system of collection and registration of data implemented at our Institute since 2002. It permits to evaluate the whole set of laboratory data coming from all the 17 diagnostic sites of our Institute distributed in Lombardia and Emilia Romagna regions.

Electron Microscopy

Negative staining EM observation was carried out on faecal contents i.e small intestine and caecum usually pooled. They were suspended in distilled water (10% v/v), shacked and then frozen and thawed twice. The supernatant was harvested and centrifuged twice (4,000 *g* and 9300 *g* for 20 min. each) for clarification. The 2nd supernatant (85 µl) was then ultracentrifuged in Airfuge Beckman for 15 min. at 21 psi (82000 *g*). The Airfuge was fitted with an A 100 rotor holding six tubes in which were put specific adapters for 3 mm grids, which allow direct pelleting of viral particles on carbon-coated Formvar copper grids. Immune electron microscopy (IEM) was performed for group A LRV and rabbit parvovirus, using specific hyperimmune sera. An equal amount (50 µl) of both the supernatant from the second centrifugation and the optimal dilution of each serum were incubated at 37°C for 1 h before being ultracentrifuged. Negative staining was finally performed using 2% sodium phosphotungstate (pH 6.8). Examination was made using a TEM Philips CM10 operating at 80 kV at 19000 to 39000 magnifications.

Genomic characterization

The RNA extraction, prediction of the VP7 and VP4 specificity by PCR genotyping and sequence analysis as well as the determination of the VP6 subgroup were those described in details by Martella *et al.* (2003; 2004; 2005).

RESULTS AND DISCUSSION

EM observation

It is here reported the prevalence of rotavirus and the other viruses detected during the period 2002-2007 by electron microscopy observation. The nsEM methods and particularly the IEM associated to Airfuge ultracentrifugation have a very good level of sensitivity (detectibility level = 10^4 particles/ml) that results, in our hand, comparable to ELISA. This type of investigation is particularly useful to detect virus involved in enteric diseases (from gut contents and faeces) which are often non cultivable (Figure 1). By EM (Table 1) viral particles were observed in 45.3% of the outbreaks; rotavirus was identified in 16.0%, coronavirus-like virus in 24.7%, parvovirus in 9% and enterovirus-like virus in 5.8% of the positive samples. Adenovirus, calicivirus and reovirus were sporadically found, and in 29 cases two or three different viruses were contemporarily observed in association in the same sample (Table 2). The comparison of the results obtained during the period 2002-2007 with the previous ones (Nieddu *et al*., 2000; Cerioli *et al.*, 2004) indicates an increase of total positivity for viruses in (1982-1999 = 36.3% and 1997-2001 = 34.1% vs. 2002-2007 = 45.3%). Looking at the prevalence of each type of virus it resulted that the presence of parvovirus and rotavirus was nearly steady while it was evident an increased positivity for enterovirus-like and, above all, for coronavirus-like. The bacteriologic and parasitologic results (Table 3) suggested that there wasn't any association between viral positivity or negativity and the presence of bacteria. In fact, similar isolations and combinations of pathogens were found either in the case of viral observations or not. In about 40% of the cases *E. coli* was the sole pathogen present and its frequency was indeed slightly higher in the absence of viral identification. More bacteria in association were frequently isolated and among the various possible combinations, the more frequently detected were, as expected and indicated by other Authors (Pisoni *et al*., 2002; Licois, 2004), *E. coli* with *Cl. spiriforme* and *E. coli* with *Cl. perfringens*. *Stapylococcus aureus* was often isolated, alone or in association with other bacteria, from skin lesions and lungs. Coccidia and other protozoa were often detected and always associated with bacteria.

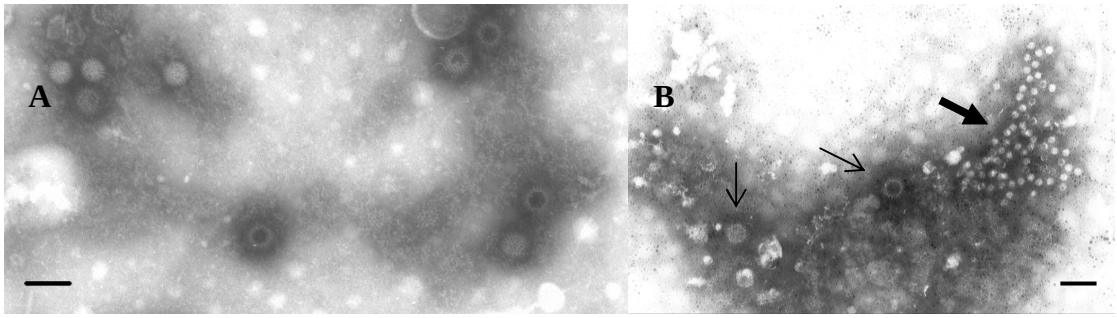

Figure 1: Microphotograph of A) rotavirus and B) rotavirus (→) in association with parvovirus (→) particles from faecal sample of diarrhoeic rabbit. NaPt negative staining. Bar =100nm

Genotyping of isolated LRV strains

Some of the LRV isolates from these diagnostic surveys were genotyped. 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 and 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 LRV strains analysed exhibited a genogroup I VP6 specificity and a long dsRNA electropherotype. However, one of the P[14],G3 strains possessed a super-short pattern. Altogether, these data highlighted the epidemiological relevance of the P[22] LRVs in Italian rabbitries.

Table 1: Cumulative results of nsEM examination and distribution of positivity for year and virus

Year	Total	Negative		Positive		Rotav.		Parvov.		Coronav.		Enterov.		Adenov.		Caliciv.		Reov.		N°
	samples	n°	%	N°	%	n°	%	n°	%	n°	%	n°	%	n°	%	n°	%	n°	%	viruses[1]
2002	35	17	48.6	18	51.4	9	25.7	5	14.3	8	22.9	2	5.7	1	2.9	1	2.9	0	0.0	26
2003	34	17	50.0	17	50.0	8	23.5	4	11.8	8	23.5	3	8.8	0	0.0	1	2.9	0	0.0	24
2004	46	27	58.7	19	41.3	4	8.7	3	6.5	12	26.1	5	10.9	0	0.0	0	0.0	0	0.0	24
2005	56	26	46.4	30	53.6	11	19.6	5	8.9	18	32.1	2	3.6	1	1.8	0	0.0	1	1.8	38
2006	47	34	72.3	13	27.7	2	4.3	5	10.7	6	12.8	1	2.1	0	0.0	0	0.0	0	0.0	14
2007	25	12	48.0	13	52.0	5	20	0	0.0	8	32.0	1	4.0	0	0.0	0	0.0	0	0.0	14
Total	243	133	54.7	110	45.3	39	16.0	22	9.0	60	24.7	14	5.8	2	0.8	2	0.9	1	0.4	140

[1]Total number of viruses observed stating the observation of several viral associations (two or more virus contemporarily presents)

Table 2: Type of associations (2002-2007)

Type of association	2002	2003	2004	2005	2006	2007	Total
Rotavirus + Coronavirus	3	3	0	4	0	0	10
Parvovirus + Coronavirus	1	2	1	0	0	0	4
Enterovirus + Coronavirus	0	1	1	1	0	1	4
Rotavirus + Enterovirus	1	0	0	1	0	0	2
Rotavirus + Parvovirus	0	1	1	0	1	0	3
Parvovirus + Enterovirus	1	0	0	0	0	0	1
Rotavirus + Calicivirus	0	1	0	0	0	0	1
Coronavirus + Adenovirus	0	0	0	1	0	0	1
Reovirus + Coronavirus	0	0	0	1	0	0	1
Rotavirus + Enterovius + Coronavirus	0	0	1	0	0	0	1
Rotavirus + Parvovirus + Adenovirus	0	0	0	0	0	0	0
Enterovirus + Coronavirus + Parvovirus	0	0	0	0	0	0	0
Rotavirus + Coronavirus + Calicivirus	1	0	0	0	0	0	1
Total	7	8	4	8	1	1	29

Table 3: Results of laboratory investigations on the base of viral presence or not (2002-2005)

Virus positivity (84)			Virus negativity (87)		
Bacteriological and parasitological negative		5	Bacteriological and parasitological negative		5
Not done		7	Not done		7
E.coli		33	*E.coli*		47
	Clostridium spp.	3		*Cl. perfringens + Bordetella*	1
	Pasteurella multocida	6		*Streptococcus* spp.	2
	Clostridium perfringens	6		*Clostridium perfringens*	4
	Staphylococcus aureus + P.multocida	1		*Staphylococcus aureus*	3
E.coli +	*Staphylococcus aureus*	1	*E.coli +*	*Salmonella* spp.	1
	Clostridium spiroforme	12		*Clostridium spiroforme*	9
	Klebsiella spp.	1		*S.aureus + P.multocida*	1
	Yersinia spp.	1		*P.multocida + Cl.spiroforme*	1
	P.multocida + Cl.spiroforme	1		*Pasteurella multocida*	1
Staphylococcus aureus		3		*Bordetella bronchiseptica*	1
Pasteurella multocida		2	*Streptococcus* spp.		1
Proteus spp.*+ Clostridium spiroforme*		1	*Staphylococcus aureus*		2
Salmonella spp.		1	*Pasteurella multocida*		1
Coccidia (always associated with bacteria)		19	Coccidia (always associated with bacteria)		22

CONCLUSIONS

The present results indicate that most cases of rabbit enteritis probably had multiple aetiologies and that the presence of viruses would not be absolutely necessary for determining enteric lesions, which on the contrary could be induced by one or more bacteria. The finding that no specific pathogens may be constantly associated with rabbit enteropathies has led the proposition that "rabbit enteritis complex" is a multifactorial syndrome, with synergic mechanisms that often enhance the pathogenicity of the various microorganism (Licois, 2004).

The use of nsEM associated to Airfuge ultracentrifugation is indeed extremely useful for detecting viral pathogens in the faecal contents of diarrhoeic rabbits. In fact, it is a quite sensitive methods that

permits to identify the different viruses, including those non cultivable *in vitro*, and to detect viral association. Among the different viruses that could be found in rabbits suffering from enteropathy, rotavirus seems to have an important but not definitive role. It is likely not able to induce primary episodes of high gravity but, acting as mild pathogen, it may become endemic. From this perspective, it may be hypothesized that under field conditions rotavirus seldom exerts direct pathogenic activity, and, more frequently, it triggers the development of bacterial infections and/or other viral pathogens. In fact, it primary causes damage on the mucosa thus predisposing the attachment and replication of bacteria. In such case it is possible 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 *E.coli*, *Clostridium* spp, coccidia and other protozoa.

The situation of intensive rabbit-breedings is characterised by intense genetic selection, exasperated productive performances, sometime overpopulation and consequently high environmental pollution of facultative pathogens. Therefore, viruses and other low pathogenic agents (es. flagellata) can explicate a more important role for the occurrence of severe enteritis in rabbit, predisposing and aggravating secondary microbial infections. On the other hand we can't exclude that the changed physiological and metabolic conditions, induced by various factors both alimentary or not, can enhance the replication of viruses normally present at a lower concentration, permitting them to explicate a pathogenic action.

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