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ELECTRON MICROSCOPY DETECTION OF VIRAL AGENTS IN RABBITS WITH ENTEROPATHY DURING THE PERIOD 1982-1999 IN ITALY

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ABSTRACT

The multifactorial rabbit enteropathy has a great importance in rabbit meat production for its economical impact. Our purposes was to estimate the prevalence of different viruses, identified by negative contrast electron microscopy, 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 period 1982-85 and 1990-99, we examined 1067 samples taken mainly (80%) from rabbits showing catharral, haemorrhagic or necrotic entero-tiflitis; the rest (20%) were showing mucoid enteropathy and caecal impaction. By EM we observed the presence of viral particles in 37.3% of them; rotavirus was identified in 41.9%, coronavirus-like virus in 25.6%, parvovirus in 21.1% and enterovirus-like virus in 10.3% of the positive samples. We also found sporadically adenovirus, calicivirus and reovirus. Often (30 cases) we contemporarily observed in the same sample two or three different viruses in association. The pathogenic role and importance as primary agent of each virus are discussed. Moreover, the availability of the results of microbiological and parasitological analysis carried out in the last triennium allowed to correlate EM observations with the contemporary presence of others pathogenic agents such as rotavirus, enteropathogenic *Escherichia coli* (EPEC) and *Staph. aureus* in sucklings rabbits; EPEC, *Cl. spiroforme* and flagellate protozoa in the others.

INTRODUCTION

In the group of diseases characterising the present situation of the Italian rabbit industry, the so-called "multifactorial conditioned diseases" are really very important. The "multifactorial enteropathy" is characterised by a great number of stress and pathogens acting in synergy, and it is the most important of these technopathies, especially in relation to its productive and economic impacts. The seriousness of this bowel pathology is related to the losses it is able to cause, either direct due to the death of growing rabbits, or indirect because of a reduced or absent growth and above all the high rate of discarded animals.

The unpredictable appearance and the clinical variability, as well as the variable pattern of pathologic lesions, make epidemiological, diagnostic and laboratory researches very difficult. Post mortem lesions are not typical and many pathogenic agents are often involved at the same time, or they follow each other, and consequently their real pathogenic role is still uncertain. On the contrary, it is well known that many conditioning factors (wrong feed formulation, decreased food taking because of adverse seasonal conditions, managerial mistakes, excessive antibiotic administration, loss of passive immunity, cold, early weaning) are involved in inducing this syndrome by promoting the overgrowing of primary or potential pathogenic agents and/or increasing rabbit sensitivity (LELKES & GHANG, 1987).

Our survey wants to estimate the prevalence of different viruses, identified by negative staining Electron Microscopy (EM), on the whole of enteric diseases analysed from the early months of 1982 to the end of 1999. That is to recognise the main features and pathogenic abilities of different viral agents and to try to attribute them an etiological role in enteric syndromes, relating their presence with pathologic lesions. Particularly, from October 1995 to

September 1996, the high rate of "mucoid enteropathy - caecal impaction (ME-CI)" (MARCATO & ROSMINI 1996; OKERMAN 1994), whose pathogenesis is still unknown (VAN DER HAGE & DORRESTEIN 1996), allowed to check in detail if known viral agents have a primary role in aetiology and pathogenesis, or if there is a "new" virus that could be regarded as a primary pathogenic agent of this disease.

MATERIALS AND METHODS

Animals and sampling

Our survey took into account the carcasses of diarrhoeic rabbits brought directly to the Diagnostic Laboratory of Brescia and there necropsied, and the pathological samples (faeces and bowel contents) coming from the Diagnostic Sections of Lombardia and Emilia Romagna, over a 18 years period. Most of the animals examined from 1982 to 1985, from 1990 to 1995 and from 1997 to 1999 had pathologic lesions related to "generic" enteropathy, which could affect only a part or the whole gut. The main clinical sign was diarrhoea, sometimes associated with bowel inflammation, excessive peristalsis or abnormal liquid secretion in small bowel. From October 1995 to September 1996, besides cases of "generic" enteropathy, we checked also animals coming from 8 outbreaks characterised by lesions referable to ME-CI. We also take into account the results of virologic tests made on diarrhoeic rabbits coming from 70 episodes of enteritis, occurred in 60 middle-large sized rabbitries, which were brought to the Diagnostic Laboratory of the Veterinary Faculty of the University of Milan, from January 1996 to June 1999.

Electron Microscopy

Negative staining EM observation was carried out on faecal samples and bowel contents (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 second 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 175 µl test 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 rabbit group A rotavirus and rabbit parvovirus, using specific hyperimmune sera. An equal amount (50 µl) of the supernatant from the second centrifugation and of the optimal dilution of each serum were incubated at 37°C for 1 hr 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 magnification.

RESULTS AND DISCUSSION

Lesions

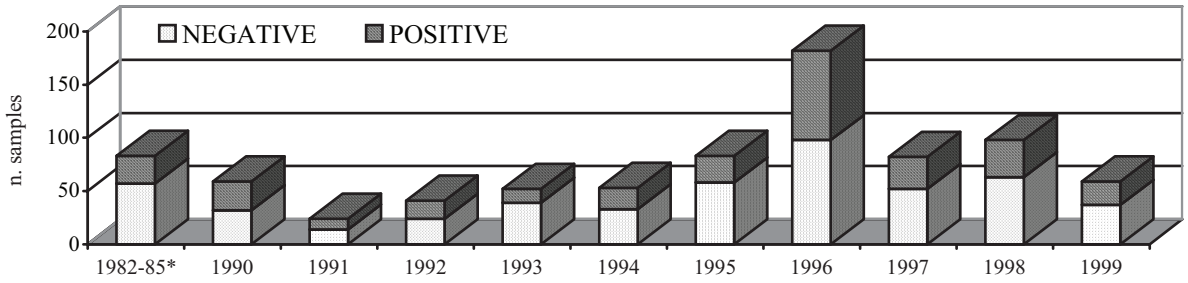
From the observations during necropsy and the data taken from anamnestic sheets two main pathological pictures could be defined. The first was found in almost 80% of rabbits of all ages (sucklings, weanlings, fattenings and breeding does), and it consisted of an enterotiphlitis, that could vary from catarrhal to catarrhal–haemorrhagic and in some cases to necrotic-haemorrhagic. The caecum was always damaged and filled with watery or liquid content. An acute involvement of small intestine and colon was frequently observed. A small part (20%) of the necropsied rabbits showed the second pathological picture that corresponds to the typical lesions of ME-CI: the caecum was constipated and filled with dry contents, the colon was empty or contained mucus, the bladder was often distended. The rabbits showing

signs and lesions referable to such form of enteropathy were sucklings, weanlings and fattenings but not breeding rabbits. They were found during the whole period considered but with a higher frequency, i.e. 50% of the examined rabbits, from Oct. 1995 to Sept. 1996.

Electron Microscopy

During the period considered we examined by negative contrast EM a total of 1067 samples. In 680 of these (63.7%) we did not found any virus, whilst in the rest 387 (37.3%) we

Figure 1: Schematic representation of the EM results during the period 1982-1999



*average value calculated over the 4 years period

Table 1: Distribution of viral positivity for year and type of virus

Year	Total	Negative		Rotavirus		Parvovirus		Coronalike		Enterolike		Reovirus		Calicivirus		Adenovirus		Total
	samples	(n.)	(%)	(n.)	(%)	(n.)	(%)	(n.)	(%)	(n.)	(%)	(n.)	(%)	(n.)	(%)	(n.)	(%)	virus
1982-85	334	230	68,9	47	14,1	30	9,0	15	4,5	12	3,6	0	0,0	0	0,0	0	0,0	104
1990	59	32	54,2	4	6,8	6	10,2	15	25,4	2	3,4	0	0,0	0	0,0	0	0,0	27
1991	24	14	58,3	3	12,5	3	12,5	2	8,3	2	8,3	0	0,0	0	0,0	0	0,0	10
1992	41	24	58,5	3	7,3	7	17,1	2	4,9	4	9,8	1	2,4	0	0,0	0	0,0	17
1993	52	39	75,0	3	5,8	3	5,8	6	11,5	0	0,0	0	0,0	1	1,9	0	0,0	13
1994	53	33	62,3	7	13,2	7	13,2	4	7,5	2	3,8	0	0,0	0	0,0	0	0,0	20
1995	83	58	69,9	8	9,6	4	4,8	6	7,2	7	8,4	0	0,0	0	0,0	0	0,0	25
1996	182	98	53,8	49	26,9	12	6,6	29	15,9	9	4,9	0	0,0	0	0,0	1	0,5	100
1997	82	52	63,4	19	23,2	6	7,3	10	12,2	2	2,4	0	0,0	0	0,0	2	2,4	39
1998	98	63	64,3	20	20,4	8	8,2	10	10,2	3	3,1	0	0,0	0	0,0	0	0,0	41
1999*	59	37	62,7	12	20,3	2	3,4	8	13,6	0	0,0	0	0,0	0	0,0	0	0,0	22
TOT	**1067**	**680**	**63,7**	**175**	**16,4**	**88**	**8,2**	**107**	**10,0**	**43**	**4,0**	**1**	**0,1**	**1**	**0,1**	**3**	**0,3**	**418**

observed at least one type of virus. Interestingly, in 30 samples all conferred between 1996 and 1998 we detected two (29 cases) or three (1 case) types of virus contemporarily. The temporal distribution of positivity is schematically reported in Figure1. The highest number of examinations was carried out during 1996, when we registered in several industrial rabbitries a sharp increase of the incidence of outbreaks of enteritis, and particularly of ME-CI, which were characterised by high morbidity and significant mortality.

The identification of virus by EM was based on their morphology and, for rotavirus and parvovirus, on immunological clumping of virions due to the use of IEM methods. The supplementary identification of rotavirus as group A has been determined since 1992 using a sandwich ELISA using specific anti-group A antisera (data not shown). Other than these two viruses we identified the following agents: coronavirus-like virus, enterovirus-like virus, reovirus, adenovirus and calicivirus. In consideration of the presence of multiple infections the total number of viral observations in the 387 positive

Table 2: Multiples infections observed at EM

Type of association	1996	1997	1998	Total
Corona + Adeno	0	1	0	1
Enterolike + Coronalike	4	1	1	6
Parvo + Coronalike	3	1	2	6
Parvo + Enterolike	1	0	1	2
Rota + Adeno	0	1	0	1
Rota + Coronalike	1	1	1	3
Rota + Coronalike + Enterolike	1	0	0	1
Rota + Enterolike	2	0	1	3
Rota + Parvo	3	4	0	7
Total	15	9	6	30

samples was 418, which are summarised in Table 1. Rotaviruses (41.9% of positives) were the most frequently diagnosed viral agents, followed by coronavirus-like viruses (25.6%), parvoviruses (21.1%) and enterovirus-like viruses (10.3%). A limited number of samples resulted positive for other viruses like adenovirus (2 cases = 0.7%), calicivirus and reovirus (1 case each = 0.2%). In most cases of multiple infections (Table 2) coronavirus-like particles (17 cases), rotavirus and parvovirus (15 cases each) were mainly involved. In particular, the most frequent association was rotavirus together with parvovirus (7 cases).

Some of the viruses here observed are well known and have been described several times in association with the occurrence of diarrheal disease in rabbits. Rabbit rotavirus has been isolated by several Authors (BRYDEN 1976; PEETERS 1984; SCHOEB 1986); it is considered as only mildly pathogenic (THOULESS 1988) and could cause important losses and high mortality only when viral infections are complicated and aggravated by secondary pathogens like enteropathogenic *E.coli* (EPEC). Based on seroepidemiological investigation rotavirus seems to be very spread (PEETERS 1984, DI GIACOMO & THOULESS 1986) and could be considered endemic in commercial rabbit populations. During the period considered the yearly presence of rotavirus varied between 5.8% on 1993 and 26.9% on 1996. The mean value was 16.4%, that is fairly lower than 35.4% obtained by PEETERS (1984) in a field survey.

The rabbit parvovirus, firstly described by MATSUNAGA (1977), has a very low pathogenicity and it is commonly isolated from the gut contents of healthy animals. It could cause very mild clinical signs in experimentally infected animals and a mild to moderate enteritis in the small intestine (MATSUNAGA & CHINO 1981). Its primary pathogenic role is still unclear but considering its frequency of identification (between 3.4% on 1999 and 17.1% on 1992, mean value 8.2%), it could be important just in multiple infections together with other infectious agents (viruses, bacteria and parasites).

Some of the other viruses detected had only a sporadic occurrence thus their pathogenic role is probably negligible. Adenovirus has been previously reported only once (BODON & PROHASZKA 1980); reovirus and calicivirus have never been described before as enteric agents of rabbits. To note that the sole calicivirus positive sample resulted negative when tested with the differential ELISA methods, based on the use of different monoclonal antibodies, which are normally employed for the detection and antigenic characterisation of both Rabbit Haemorrhagic Disease Virus (RHDV) and Rabbit Calicivirus (RCV) (CAPUCCI L., personal communication).

The observed frequencies of coronavirus-like virus and enterovirus-like virus were quite high and suggestive of a potential pathogenic role. Coronavirus-like particles have been demonstrated in rabbit with diarrheal disease (LAPIERRE 1980; OSTERHAUS 1982) and a high prevalence has been found in seroepidemiological surveys (DEEB 1993), indicating a wide diffusion in rabbitries. The oral inoculation of such particles into weanling rabbits can cause mild diarrhoea (DESCOTEAUX & LUSSIER 1990). Since we did not test the coronavirus-like positive samples (10% of examined samples) with reference sera we can not be completely sure that they are antigenically related to RECV. However, at least two data support such final classification: 1) the morphology of virions, pleomorphic and roundish particles of 45-170 nm of diameter, with short and not clearly defined surface projections, closely resembling those described in literature, and 2) the positivity of several samples to the haemoagglutination test with rabbit red blood cells (data not shown). Such particles were often already clumped with an evident fuzzy halo due to the presence of precipitating antibodies. This meant that at the moment of death the coronaviral infection was probably already in a sub-acute chronic phase and that coronavirus-like particles could likely act as a factor favouring the replication of secondary bacteria more than primary cause of fatal enteritis.

The term enterovirus-like virus is extensively used to define all those roundish, 28nm in size, nude and smooth virions, commonly observed by EM in various commercial species, in due

course of enteric diseases primarily caused by other infectious agents. For this region they could be considered a sort of "bioindicator" of the intestinal anatomic and functional integrity. In our survey their presence varied between 0% on 1993 and 9.8% on 1992 (mean value 4.0%), and they were often found in association with other viruses (12 cases out of 43 totally observed = 27.9%). Indeed, can not exclude that these particles correspond to the picobirnavirus (GALLIMORE 1993), stating the strict morphological similarities existing with this group of non cultivable RNA viruses, identified in several species (humans, pigs, chickens, guinea pigs) including rabbits. LUSERT (1995) found that picobirnavirus were commonly excreted by 10% of rabbits without causing any symptom or lesions.

Table 3: Results of laboratory examinations from 8 outbreaks of mucoid enteropathy - caecal impaction.

Type of examination	N. samples	N. positives	Details on positivity
Virology	36	33	24 rotavirus; 7 coronavirus-like; 2 parvovirus
Bacteriology	8 units	8 units	5 *E.coli* (1/08, 2/0103, 2/not typed) 3 *Pseudomnoas* sp. 2 *Pasteurella multocida* 1 *Staphylococcus aureus*
Parasitology	36	1	*Trichomonas* sp.
Histopathology	18	18	oedema (100%), necrosis of villi (30%); inflammatory cell infiltration (80)

The result of laboratory examinations conducted on samples taken in 8 outbreaks of ME-CI (Table 3) were used for better understanding the role of viruses in the aetiology of this syndrome. Rotavirus was the predominant virus, being present in most but not all subjects, mainly in the caecum and frequently at a low concentration. In one outbreak we did not find any virus at all, whilst we isolated in all the outbreaks pathogenic bacteria like EPEC and *Staphylococcus aureus.*

Table 4: Laboratory investigation on samples conferred to the University of Milan in the period 1/1/96 -30/06/99.

Agent	<28dd	29-40dd	41-60dd	adult	Total
Rotavirus	22,2%	20,0%	15,4%	0,0%	18,0%
Coronavirus-like	5,5%	0,0%	0,0%	0,0%	2,0%
Enterovirus-like	0,0%	6,6%	5,0%	0,0%	2,0%
E. coli	53,5%	52,0%	66,0%	75,0%	57,6%
of which EPEC	23,8%	24,0%	44,5%	16,7%	29,3%
C. spiroforme	22,5%	54,3%	66,3%	25,0%	46,5%
S. aureus	17,5%	0,0%	0,0%	0,0%	5,3%
Yersinia pseudotubercolosis	0,0%	0,0%	0,0%	8,3%	0,4%
Other bacteria	2,5%	33,3%	27,3%	16,7%	23,3%
Flagellata	0,0%	20,0%	20,5%	14,3%	13,7%
Passalurus ambiguus	0,0%	0,7%	1,0%	10,7%	1,1%
Cysticercus pisiformis	0,0%	1,5%	0,0%	0,0%	0,5%
Eimeria spp.	*	0,7%	*	*	0,3%

* less than 5 oocysts for field (400x)

Moreover, we got more indications on the correlation and synergism existing between viruses and other infectious agents, by analysing the results of the laboratory investigations on 600 samples from 70 outbreaks of enteritis (GALLAZZI 1999) (Table 4). Several bacteria and parasites in association, sometimes at a very high titre were isolated and their frequency varied according to the age. In particular we must underline the rare detection of *Eimeria* sp., the very high prevalence of *C. spiroforme* among weanlings and fattenings, the constant occurrence of EPEC infections in rabbits of all ages and the significative presence of flagellata *(Chilomastix cuniculi, Monocercomonas cuniculi).*

CONCLUSIONS

A first comment regards the utility of negative contrast EM examination for the rapid identification of virions in course of enteroptahy, because it permits the contemporary observation of different viral agents, both singular or in association, and the identification of

viruses hardly or absolutely not cultivable in vitro. Indeed, the methods here used in previous studies proved to be as sensitive as immunoenzymatic tests and to have a detectivity limit of 10^3-10^4 particles/ml (LAVAZZA A., personal observations). Besides, same aspects already known in literature, concerning rabbit enteropathy are confirmed on the basis of the results of our investigation:

1. The enteritis complex of intensive rabbit-breeding has an aetiology determined by many different factors, in which pathogenic agents (EPEC) and potentially pathogenic ones *(C. spiroforme,* other bacteria*,* flagellata protozoa and enteric viruses) can appear contemporary in the same subject.
2. Viruses, and rotavirus particularly, should not be able to induce primary episodes of high gravity but, acting as mild pathogens, should have the capacity of became endemic. The situation of intensive rabbit-breeding, is characterised by intense genetic selection, exasperated productive performances, overpopulation and consequently high environmental pollution of facultative pathogens. Therefore, these viruses and others agents considered less or not at all pathogens, as flagellata protozoa of genus *Chilomastix* and *Monocercomonas,* 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 to enteric level 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.
3. The arising of cases of ME-CI, observed in Italy during the period 1995-1997 in several commercial rabbitries, and now no more indicated as a problem, is not explainable with the appearance of "new" viral or bacterial agents with an enteric replication. Therefore, other factors not yet identified could be involved in causing or predisposing rabbits to this disease.

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