Proceedings of the

4-7 July **2000** – Valencia Spain

These proceedings were printed as a special issue of WORLD RABBIT SCIENCE, the journal of the World Rabbit Science Association, Volume 8, supplement 1

ISSN reference of this on line version is 2308-1910
(ISSN for all the on-line versions of the proceedings of the successive World Rabbit Congresses)

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Volume B, pages 187-194

EPIZOOTIC ENTEROCOLITIS OF THE RABBIT: REVIEW OF CURRENT RESEARCH

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HISTORY AND DESCRIPTION

A new and serious gastrointestinal syndrome has appeared in rabbit breeding colonies in the West of France since the end of 1996/beginning of 1997. The disease is characterized by small quantities of watery diarrhoea following a decrease in feed intake, has high mortality rates (30 – 80%) and spreads very rapidly. It spread to other regions of France in 1997 and 1998 (Duval, 1998) and in Europe (Lebas and Coudert, 1997). Usual antibiotic treatments have been completely ineffective. It was only in mid-1998 that mortality began to be controlled, after establishing very strict hygiene and sanitation measures, combined with the use of certain antibiotics (bacitracin, tiamulin). At the present time it is estimated that 90 – 95% of breeding colonies are or have been affected by rabbit epizootic enterocolitis (ERE), whatever the rabbit race and strain. The disease mainly affects young fattening rabbits, between six and eight weeks of age. The problems usually occur after weaning but have also been observed in adult rabbits. Comparatively with other epizootic diseases (myxomatosis, viral haemorrhagic disease), wild rabbits do not seem affected. Nevertheless, ERE have been observed in reared wild rabbits.

ERE can be distinguished from other usual rabbit intestinal diseases by the specific clinical pattern and lesions. In addition to the diarrhoea and mortality, there is usually pronounced abdominal swelling due to dilatation of all segments of the gastrointestinal tract, including the stomach, the contents of which are very liquid. These symptoms are sometimes associated with caecal paresis and the presence of mucus, especially in the colon and sometimes in the small intestine (Coudert *et al*, 1997). However, there are no macroscopic congestive or inflammatory lesions, particularly in the caecum, whereas this is the usual site of typical lesions of acute enteritis (congestive features of mucosa, hemorrhagic suffusion, typhlitis, etc) in cases of caecal coccidiosis, clostridiosis, klebsiellosis and colibacilloses caused by enteropathogen *Escherichia coli* (Licois, 1998). There are, on the other hand, many similarities with a disease previously described as mucoid enteritis or mucoid enteropathy (Muir, 1943; Hurt, 1949; Hagen, 1956; Van Kruinignen and Williams, 1972). According to these reports this disease decimated large numbers of colonies, particularly in Great Britain and the United States, almost the only countries where this disease was studied, apart from Hungary (Vetesi, 1970). The above authors described high mortality, with particularly high expression of the disease between 7 and 10 weeks of age, excessive thirst, distension of the abdomen and dilatation of the entire gastrointestinal tract, caecal paresis in some cases and, of course, passing of mucus. There was no change in body temperature. The absence of macroscopic and histologic lesions, apart from hyperplasia of mucous cells throughout the small intestine, is also a feature emphasized by Van Kruiningen and Williams (1972). This is why the term mucoid enteropathy was used in place of mucoid enteritis, because there was no visible inflammation of the intestine (Flatt et al, 1974). The difference between these old descriptions and the actual situation lies in the sporadic nature of the cases described whereas ERE, which has been rampant in Europe for more than three years, corresponds to true

epizooty. It is, however, true that the conditions of rabbit production and marketing networks are no longer comparable.

The first research studies, coordinated by the Association Scientifique Française de Cuniculture, were undertaken in the second half of 1997. The aims were **i/** to research alimentary factors which might have a direct effect or predisposing effect on the development of ERE, **ii/** to reveal the possible involvement of one or several pathogens in the genesis of ERE and to identify the(se) agent(s) and **iii/** to set up an investigation to follow the development of the disease in the field. The first reports on these studies were presented during the 7th Journées de la Recherche Cunicole in Lyons in 1998 (Duval, 1998; Lebas, 1998; Le Gall *et al*., 1998; Licois, 1998) and then in Paris (Licois and Coudert, 1999).

Foodstuffs were the first to be suspected. The studies performed by F.Lebas and his colleagues (INRA, Toulouse) focused on several aspects, including the nature and levels of principal ingredients, premix, mycotoxins, pesticides (Maverick, Karaté, Gaucho). All these hypotheses were eliminated. Studies of gastrointestinal transit did not show any differences between feeds suspected of reproducing ERE and the same feeds treated by irradiation. Three years later, everything confirms that feeds were not the direct cause. However, it can be a passive vector. Feed taken from a feeder in a contaminated breeding colony may transmit the disease. Virulence of contaminated feeds appears to be conserved no longer than 3 to 4 months.

The first results in the second round of rapidly set up studies showed that the disease associated with the above symptoms was **reproducible** and **contagious** (easily transmitted to contact animals), confirming the existence of one or several infectious pathogens. Several arguments supported a viral hypothesis in ERE, particularly the epizootic nature and the diffusion of the pathogen, the transmissibility of the disease and the existence of histologically-proven lung lesions suggesting a viral infection (Wyers, 1998). In addition there was the ineffectiveness of the majority of common antibiotics. Bacitracin and tiamulin also have a limited effect in that the disease recurs within a week of ceasing treatment, even if treatment had been constant for 5 or 6 weeks. On the other hand, usual **bacteriological investigations** have not revealed a univocal response concerning the direct involvement of a bacterium, whether aerobic-anaerobic flora or anaerobic flora.

The following is a review of the results on researches performed in various directions and obtained during the past two years.

EXPERIMENTAL REPRODUCTION AND RESEARCH FOR EFFECTIVE VIRULENT MATERIAL

From the outset, one of the first difficulties was to find an inoculum with a positive response in our animals (EOPS rabbits). In fact EOPS rabbits are an experimental tool of choice to overcome interference from all pathogens other than the one being studied. We showed that experimental reproduction of ERE with the animal model was usually expressed by morbidity and a relatively low mortality rate (10-15%) whereas the mortality in ordinary rabbits was higher than 60%. Of the 5 samples from the gastrointestinal tract taken at that time from breeding animals with all the characteristics of ERE, only one was able to reproduce the disease in our animals without ambiguity. This sample was used for all the studies in 1998 and the first half of 1999.

From the beginning of 1998 the disease was accurately reproduced on intestinal samples taken from experimental animals, dead or diseased, which eliminated other hypotheses (feeds, toxins, pesticides, mycotoxin, etc). Using EOPS rabbits it was possible to describe the specific clinical and lesion features of ERE, which distinguished it from the symptoms and

lesions of other known intestinal affections. They were the same as those observed on the field. Animals are bloated with watery diarrhea of low intensity. Gross lesions are mainly a distension of the whole intestinal tract including the stomach which is fill with gas and fluid. No inflammation or congestion of the intestine are visible. These symptoms are associated with caecal paresis and the presence of mucus, especially in the colon, in 40 to 60% of cases. The kinetics of ERE have been established, with a peak at 4-5 days post-infection. ERE has also been experimentally reproduced from contaminated foodstuffs, by contact between animals and also by contact with contaminated breeding equipment (cages, feeders, etc), even when carefully washed but not disinfected. Finally, two trials showed the possibility of reproducing the disease from lung extracts. This finding, which must be confirmed, is fundamental because, in combination with other observations, it reinforces the viral hypothesis.

Two trials have also been performed on "immunity". The rabbits surviving to a first inoculation with infective digestive material became resistant to a challenge with the same product.

All these studies have clearly demonstrated the role of an infectious agent in the development of the disease.

From the methodological point of view, ERE is regularly reproduced from samples taken from intestinal content of diseased animals and frozen. Infectivity of these samples is conserved after several months at –20°C. This result is important from the practical point of view: the same inoculum can be used for several trials. We also sought to improve the response of EOPS rabbits to an inoculum which reproduced the clinical features of ERE. This was possible by artificially reducing the immune defences of the animals (Licois *et al*., 1998). Mortality was close to 70% in EOPS rabbits treated for almost 3 weeks with corticosteroids (dexamethasone) whereas it was 14% in the non-treated control group. Moreover, the mortality was earlier and the ERE lesions could even be observed 24 hours after inoculation. This animal experimental model seemed therefore to constitute an interesting development for subsequent studies on ERE, especially by providing greater quantities of biological material for the constitution of a reference inoculum or to set up purification techniques. Unfortunately, subsequent experiments have not all met expectations, particularly because of the lengthening of protocols and this high mortality is not systematically reproduce.

In collaboration with AFSSA at Ploufragan, we showed that ERE could be reproduced from samples removed from dead animals as early as 2 days after inoculation of the donors. Similarly, it was shown that the infectivity of samples was conserved after limited dilution (1/10), which suggests that the concentration of the pathogen is low in the intestinal content. We were able to verify in another trial that samples taken from infected rabbits not expressing ERE were able to reproduce the disease in animal recipients.

Further improvement in the model was obtained by experimenting with the modes of administration of the inoculum: oral inoculation (in drinking water or aerosol administered on food or on the nose) was more effective than inoculation directly into the stomach (esophageal catheter). The reason might be that there is a primary cycle of the pathogen in the upper gastrointestinal tract. Buccal inoculation achieved 30 to 40% mortality.

It was also necessary to widen the representativeness of samples tested in the laboratory in relation to the field. Other samples were therefore tested, one of which was from suckling animals from one breeding colony. They died at 21-26 days with clinical symptoms of ERE. It was possible to transmit the disease to weaned EOPS rabbits with this biological product.

The effect of age was also analyzed and no differences in sensitivity of EOPS rabbits were found between 3 and 10 weeks of age.

OTHER STUDIES

HISTOPATHOLOGY STUDIES

In collaboration with the National Veterinary School of Nantes (Pr. M. Wyers), a large histopathology study was undertaken on immunodepressed (corticosteroids) and non-immunodepressed, and inoculated and noninoculated EOPS rabbits. Sequential sacrifice was performed on animals at different stages of infection (with or without symptom of ERE) and various organs were sampled to provide improved characterization and definition of development site of the pathogen. The results were as follows:

- mainly epithelial lesions of the small intestine were particularly marked towards the distal region (ileum) and characterized by proliferation of glands, marked generalized atrophy of villi and features of epithelial degeneration and necrosis, often slight and disseminated.
- Epithelial lesions of the large intestine were mainly degenerative and slight.
- Signs of inflammation were moderate and not constant.
- There were diffuse lesions of interstitial pneumonia, of varying intensity.
- No lesions were detected in other organs (liver, spleen, mesenteric ganglia, thymus, heart, kidneys, suprarenal gland) apart from those related to corticosteroids in immunodepressed rabbits.

These lesions were above all evident in immunodepressed rabbits. They were more marked in rabbits with external clinical signs of ERE. The features of the lesions in the small intestine strongly suggested an infectious viral agent responsible for predominantly epithelial lesions leading to glandular abnormalities and considerable reduction in the absorbing surfaces of the villi, which could have explained the clinical signs. The lesions were not specific; several viruses cause this type of lesion or similar lesions, and in many other animal species. The pulmonary lesions must be interpreted with caution because lesions of the same type were found in healthy controls, although less marked.

BACTERIOLOGICAL INVESTIGATIONS

Among the nucleic sequences identified from extract of intestinal content, *Yersinia enterocolitica* occurred several times. Although the symptoms and clinical signs of the disorders caused by this bacterium are very different from those of ERE, a study was nevertheless undertaken on this bacterium. Twenty samples from different animals and various inoculums were seeded on selected media. The bacterium was never isolated.

Moreover, some of the most representative samples from rabbitries with ERE were sent to Dr. Popov (Institut Pasteur) for further studies on anaerobic bacteria, particularly *Clostridium*. This study showed that the results were not uniform. Three quarters of the samples revealed only *Clostridium perfringens*. C. sordelli was identified in the remainder. Counts were consistent with disease. However, the *C. sordelli* strain was not toxinogenic (there were no genes coding for the lethal toxin in mice). Genes coding for alpha, beta2 and theta toxins were revealed for *C. perfringens*, but these toxins are more often involved in toxic alimentary infections and cause necrotic lesions not observed in ERE. Investigations for *Bacteroides fragilis* were negative for all samples.

Similarly, Pr. A. Milon (National Veterinary School of Toulouse) has analyzed 5 isolates of *E. coli* O132 and O85, serogroups frequently recovered in ERE He tried to identify the genes of virulence belonging to the locus of enterocyte effacement (LEE) which is present in all the pathogenic strains of the rabbit, studied for the moment (O103,O26, O15, O109, O128, O132) and absent in non pathogenic strains. Neither the gene *eae* nor the genes *espB* and *rorf1*, which are characteristic of the LEE, were found.

RESEARCH FOR THE PATHOGEN

Several approaches were used together and separately to identify and isolate the pathogen, including concentration and purification on density gradient, electronmicroscopy, cell culture and molecular biology.

Cell cultures

Four rabbit cell lines were initiated to study the possible cytopathogenic effects of the samples available. After lengthy studies to culture and then infect with various inoculums, no significant result was obtained. The main difficulties stemmed from the complete lack of knowledge of the biology of the pathogen (target organs, evolution of viral burden, etc.) and therefor the use of an inappropriate cell system. This approach was abandoned when we had enough intestinal material to establish purification techniques by ultracentrifugation.

Purification and Microscopy

One of the most tangible points in electronmicroscopy (EM) which supported the priority we gave to exploring a viral cause, was the observation in 1998 of relatively homogeneous particles in one of the fractions after purification of gastrointestinal extracts from diseased animals and which was not present in healthy animals. These particles, which suggested RNA enveloped viruses, were however found in small and variable quantities according to the number of days post-inoculation (after experimental infection) but also according to each animal on any given day. Various methods were tried in order to increase the purification yield: precipitation (20% saccharose cushion, polyethylene glycol, ammonium sulphate), then separation by velocity and/or density gradient (saccharose, potassium tartrate-glycerol, cesium chloride). Precipitation using ammonium sulphate combined with separation on saccharose density gradient provided the best fractions. To date more than 30 samples have been treated (gastrointestinal and pulmonary extracts from healthy and diseased animals at different time points of the disease, on inoculums of different origins, etc). However EM after negative staining was disappointing each time because there were too few particles to conclude with certainty.

Research into genome structure

Two libraries of cDNA were achieved from RNA extracted from purified fractions (the richest according to EM observation). A total of 6,000 clones were obtained. Of these 120 had a fragment greater than 700 base pairs inserted and were sequenced and then compared with nucleic sequences of known organisms present in international databanks. No correlation was found between the viral sequences of databases and those of recombinant clones. On the other hand, analogies were revealed with sequences from rabbit, phages or bacteria.

In view of the disappointing results from this approach, which might be due to low amounts of virus in samples, a further approach was planned. We started to screen for known enterotropic virus by using specific oligo-nucleotide primers. Non-pathogenic calicivirus of intestinal tropism has been described in the rabbit (Capucci *et al.,* 1996). But all the samples tested from animals with ERE were negative for calicivirus. However, using a similar approach, we were able to identify, isolate and cultivate a rotavirus from a sample originating from a breeding colony with ERE. Several tests have been positive for rotavirus in many of our samples. Rotaviruses have also been described in the rabbit. They usually lead to only moderate disease during the period around weaning (Thouless *et al.,* 1988). It is therefore necessary to determine whether the strain which we have isolated is able to reproduce ERE lesions.

Using similar methodology, several other families of viruses are being studied at AFSSA (Ploufragan, France) (Parvovirus, Circovirus, etc), although to date there have been no positive results.

CONCLUSION

ERE continues to be rampant in the field (re-emergence of the disease in 1999). The more or less good control of the disease, particularly of mortality, has been achieved by better animal raising standards (single batch management, sanitary isolation, hygiene safeguards…) but also, at the moment, by treatment with antibiotics. Recently Macchioni et al. (2000) have mentioned, in an open-air small rabbit unit without treatment before and during the course of ERE, that the disease does not disappear if nothing is done. The socio-economic situation is difficult for breeders and justifies continuing the research. We believe that attention should still be focused on viruses and this is the approach with which we shall continue. But this does not exclude the possibility that other approaches continue to be explored in collaboration with other researchers, particularly bacteriologists.

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