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EXPERIMENTALLY INDUCED EPIZOOTIC RABBIT ENTEROPATHY: CLINICAL, HISTOPATHOLOGICAL, ULTRASTRUCTURAL, BACTERIOLOGICAL AND HAEMATOLOGICAL FINDINGS

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Abstract: Epizootic rabbit enteropathy is an emerging disease that has appeared in French intensive enclosed rabbit farms since the beginning of 1997. Common clinical signs are mild watery diarrhoea with considerable distension of the abdomen. At necropsy, a significant dilation of the stomach and small intestine without gross evidence of acute or chronic enteric lesions (inflammation or congestion) was observed. The purpose of this study was to describe the anatomopathologic changes concerning the small intestine and those concerning the blood profile, in experimentally infected rabbits. In a first part of the experiment, thirty animals were inoculated with a reference inoculum and five were kept as controls for clinical signs examination and histopathological study. In a second part, 17 out of the inoculated rabbits and the 5 controls animals were randomly assigned to blood testing. Microscopic lesions were studied in sections from the different parts of the small intestine (duodenum, jejunum and ileum) by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The histological findings revealed only limited inflammation in inoculated animals. Major villous changes were atrophy, fusion, destruction and loss of epithelial cells. In inoculated rabbits, the congestion and dilation of blood vessels of jejunal *lamina propria* were significantly higher than in control animals ($P<0.005$). There was significantly more ($P<0.05$) apoptosis of cells of the jejunal epithelium in inoculated rabbits than in control animals. Infiltration of polymorphonuclear neutrophiles was observed into the jejunal or ileal *tunica muscularis.* SEM performed on the intestinal tract of 15 inoculated rabbits revealed blankets and globular particles of mucus associated with numerous bacteria on jejunum and ileum villi. This was not observed in the intestinal tract of control rabbits. Bacteria were found adhering to the epithelial surface and inside intestinal epithelial cells in a few animals by TEM and by light microscopy after Warthin-Starry staining. None of the bacteria isolated from the intestinal mixed contents and cultivated on usual media, are commonly known as rabbit's pathogens. Regarding the haematological profile, neutrophil counts significantly increased ($P<0.05$) and lymphocyte counts significantly decreased ($P<0.01$), in inoculated rabbits compared to those of the control group.

Key words: Epizootic rabbit enteropathy, *Oryctolagus cuniculus*, electron microscopy

INTRODUCTION

Digestive disorders are a major cause of losses among broiler rabbits. It is estimated that these disorders account for about 10-12 % of mortality in intensive rabbit farms in France (Koehl, 1997) and consequently cause considerable economic losses. The most commonly isolated digestive tract pathogens were *Eimeria* spp, enteropathogenic *E.coli* strains (mainly serotype O15: K-:H- and

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O103:K-:H2) presenting "eae" gene, *Clostridium spiroforme*, *Clostridium piliforme* (*Bacillus piliformis*) and *Pasteurella multocida* (Hinton, 1977; 1979; Devos *et al.*, 1980; Devos, 1981, 1983, 1985; Sanyal and Gopikrishna, 1988; Maire, 1989; Hoop *et al.,* 1993; Bhasin and Singh, 1995; Blanco *et al*.,1996).

At the end of 1996, a new clinical syndrome has emerged in French intensive enclosed rabbit farms. This disease was first named Enterocolitis and is now called Epizootic Rabbit Enteropathy (ERE) (Le Gall *et al.*, 1998; Licois, 1998; Licois *et al*., 1999). This disease has spread from France to other countries and is now endemic on the European continent. The mortality rates at the onset of the epizooty were high (30 to 80%) (Licois *et al.*, 1998; Marlier and Vindevogel, 1998). During outbreaks, rabbits aged from 6 to 14 weeks first stop drinking and later stop eating. The affected rabbits show a distended abdomen ("watery belly") with mild diarrhoea of weak intensity. The macroscopic findings at necropsy reflect the clinical signs. The stomach and small intestine are distended with gaseous and watery contents. The caecal content may be either watery or impacted. In the large intestine, there may be either no lesion at all, or the presence of mucus may be observed (Coudert *et al.*, 1997; Licois *et al.,* 1998, 2005). Sporadic cases may sometimes be observed in older or in suckling rabbits just before weaning (Licois, 1998; Licois *et al*., 2005). The disease has been reproduced several times by inoculation of intestinal contents from field cases (Licois *et al.*, 1998; Licois and Coudert, 2001; Licois *et al*., 2005). In spite of numerous experimental studies, the aetiology of ERE is still unknown. Dietary and food poisoning implications have been ruled out (Lebas, 1998 ; Licois and Coudert, 1999), while viral causes, especially involving rotavirus, seem very questionable (Cere *et al*., 2000; Licois *et al*., 2005; Szalo *et al*., 2007). Nevertheless, the trials carried out to reproduce the disease suggest an infectious origin (Lebas *et al.*, 2001; Le Gall *et al.*, 1998; Licois, 1998; Licois *et al.,* 2005). The aim of this study was to describe the sequential microscopic and ultrastructural lesions of the digestive tract but also the haematological profiles of diseased rabbits in order to gain information on the aetiology of this syndrome.

MATERIALS AND METHODS

Animals, inoculum, experimental design

Thirty-five 6-weeks-old male New Zealand specific pathogen free rabbits (Charles River Laboratories Belgium) were used (mean weight 635 g). They were housed and maintained under standard conditions. They received drinking water *ad libitum* and a commercial pelleted food without coccidiostat or antibiotic. The temperature of the rabbitries was kept at 20°C, with a light/dark cycle of 12 h.

In a first part of the experiment, dedicated to clinical, histopathological and bacteriological investigation, 30 rabbits were inoculated *per os* with 800 µl of infective material (TEC) provided and described by Licois and Coudert (2001) and Licois *et al*. (2005). The most important feature of TEC is the absence of bacteria such as *E.coli* and parasites such as *Eimeria* spp (Licois *et al*., 2005). Five rabbits were kept as control animals and were housed separately to prevent cross-contamination. Seven animals (one control and six inoculated) per day were sequentially killed at 3, 4, 5, 7 and 9 days post infection (DPI). In the second part of the experiment, 17 out of the inoculated rabbits and the 5 controls animals were randomly assigned to blood testing.

Clinical assessment and necropsy

The animals were observed twice a day for at least 10 minutes and weighed once every day. The clinical signs typical for ERE such as abdominal distension, diarrhoea and anorexia were checked on a daily basis. A complete post-mortem examination was performed on all rabbits. All care and experimental procedures were approved by the animal care and ethics committee of the Faculty of Veterinary Medicine, University of Liège (N° 144).

Histopathological examinations

Intestinal samples (1 cm long) from the proximal and distal parts of the duodenum, jejunum and ileum were collected from all inoculated and controls rabbits. They were fixed in neutral buffered 10% formalin and paraffin embedded. Tissue sections were cut at 5 µm and stained with haematoxylin and eosin (HE). Sections were observed by light microscopy and the following parameters were scored: inflammatory reaction in the *lamina propria*, presence of cellular debris and bacteria in the lumen of the intestine, presence of apoptotic enterocytes in crypts and villi, dilation and congestion of blood vessels in the *lamina propria* and submucosa. For each of these parameters six high-power fields (HPF) from each section were counted at a magnification of 400× and a score was given from 0 to 3 (0: absence or normal; 1: mild; 2: moderate and 3: severe). The *lamina propria* of the duodenum, jejunum and ileum was examined to determine the number of infiltrated polymorphonuclear leukocytes (PMN). A score was given based on the number of PMN and presented as follows: score 0: 1-2 PMN/HPF (normal); score 1: 2-10 PMN/HPF (mild); score 2: 20-50 PMN/HPF (moderate), score 3: ≥50 PMN/HPF (severe). To determine the amount of apoptotic enterocytes, both the number of apoptotic enterocytes and the total number of enterocytes per villus were recorded and an average percentage was then calculated. For the apoptotic cells, the following scores were noted: score 0: 1-2% of enterocytes affected, score 1: ≥10%, score 2: ≥30 % and score 3: ≥50%. For the bacteria observed in the intestinal lumen; score 0: <10 %, score 1: 11-20%; score 2: 21-30% score 3: 31-59%, score 4: ≥60% of the area of the HPF. The alterations to the blood vessels in the *lamina propria* and the *submucosa* were scored by the following scale: 0= normal aspect, 1= slight dilation, 2= marked dilation and congestion, 3= severe congestion and extravasations of red blood cells.

Dilation and congestion of blood vessels scores and apoptotic enterocytes scores were analysed by Fisher's exact test (Statistica 6.1, Statsoft, Inc, 1983-2003). From the intestinal samples showing evidence of intra- or intercellular bacteria in the TEM screening (jejunum and ileum of 3 rabbits), additional sections paraffin were cut and stained with Gram, Giemsa, Periodic Acid Schiff (PAS) and Warthin-Starry techniques and further observed by light microscopy.

Ultrastructural examinations

Different parts of small intestine (ileum and jejunum) of 15 infected animals, selected on 3, 4, 5, 7, 9 DPI and of two controls rabbits sacrificed at 3 and 4 DPI were examined by scanning electron microscopy (SEM). The cellular infiltration by bacteria was studied by transmission electron microscopy (TEM). Tissues collected during necropsy were fixed in 2.5% glutaraldehyde solution in 0.1% cacodylate buffer (pH 7.2) during 24 hours. They were then post-fixed in 1% osmium tetroxide in MilliQ water.

For SEM, the samples were dehydrated in increasing concentrations of ethanol and finally in acetone. Afterwards the samples were dried by critical point drying in carbon dioxide. They were glued with carbon cement on aluminium subs and coated with gold in a vacuum evaporator. Observations were made using a Philips SEM501 scanning electron microscope (FEI, Eindhoven, The Netherlands). Analogue images were converted to digital images (8 bit, 2800 × 34800 pixels) using the Orion Frame Grabbing for SEM (E.L.I. sprl, Brussels, Belgium).

For TEM, fixed samples were incubated overnight at room temperature in freshly prepared 2% uranyl acetate solution in MilliQ water and dehydrated in increasing concentrations of ethanol and finally, in anhydrous propylene oxide. After embedding in Spurr medium, the specimen blocks were trimmed to obtain a cutting face of 0.5 × 1 mm^2 to 1 × 2 mm^2, and ultra-thin sections in the gold to mat sliver interference colour range were cut using an ultracut microtome and placed on pioloform-coated copper grids (150 mesh). The sections were stained with lead citrate for examination using a Philips EM208S transmission electron microscope (FEI, Einhoven, The Netherlands).

Bacteriological examinations

The bacteriological examinations were performed on mixed intestinal contents (jejunum and ileum) of the animals utilised for SEM and TEM observations. The mixed intestinal contents were collected before fixation of the intestinal sections in glutaraldehyde. They were plated onto Sheep-blood agar plates and MacConkey agar plates (OXOID, Drongen, Belgium) and onto Shaedler agar plates (OXOID, Drongen, Belgium). Plates were incubated at 37°C for 24 to 48 hours in aerobic and anaerobic chambers (MK3 Anaerobic Work Station, Led-Techno). The isolated strains were identified and based on colony morphology, Gram's stain, biochemical reactions and polymerase chain reaction for "*eae* A" intimin *E. coli* gene (China *et al*., 1996). For *E. coli* a sero-biotyping was performed according to the technique described by Peeters *et al* (1988). Bacteria of particular interest were identified to the genus and species level with Api 20E, Api20 NE and Rapid ID 32 A (Biomerieux, Belgium).

Haematological examinations

Blood for serum chemistry determination was taken from jugular vein by mean of tubes venojet (3 ml) with added EDTA (TERUMO). Blood analyses were performed at the Department of Biochemistry of the Veterinary Faculty Liège (Belgium) with automated haematology analysers involving veterinary software (Cell dyn 3500, Abbott). Dynamics of haematological indices (erythrocytes, haemoglobin, haematocrit, differential and global leucocyte counts (10^9/litre, neutrophils, lymphocytes, basophils, monocytes and eosinophils) were studied. Haematological values were determined on day 3 before inoculation and from day 3 to day 9, after inoculation. The rabbit's haematological profiles were compared by the Mann-Whitney test for unrelated samples (Statistica 6.1, Statsoft, inc, 1983-2003).

RESULTS

Clinical findings and gross examination

The clinical signs and results of post-mortem examinations shown in Table 1. A mild watery diarrhoea and severe abdominal distension were the most obvious clinical signs observed in inoculated rabbits.

Table 1: Clinical signs and gross lesions in inoculated animals

Days post-inoculation	Case No	Clinical signs	Gross lesions
3 (n=6)	11, 16, 17, 18, 20	AS, D	+++
	23	~	++
4 (n=6)	3, 8, 9, 26, 27, 29	AS, D	+++
5 (n=6)	2, 4, 13, 21	AS, D	+++
	5	AS, D, M	++++
	15	AS, D	+++ (IF)
7 (n=6)	7, 28	~	+++
	14	AS, D	+++
	6	~	~
	10, 12	~	++
9 (n=6)	1	AS, D	++
	19	~	+++
	22	~	~
	24, 25, 30	~	++

AS, abdominal swelling; D, diarrhea; (IF), inflammatory lesions; M, mucus; +, liquid content in the stomach only; ++, liquid; content in stomach and in limited segments from small intestine; +++, liquid contents of the whole digestive tract (caecal content liquid or impacted); ++++, liquid contents of all digestive tract (caeca liquid or impacted) and presence of mucus in the colon; ~, no clinical signs or lesion; n, number of inoculated animals

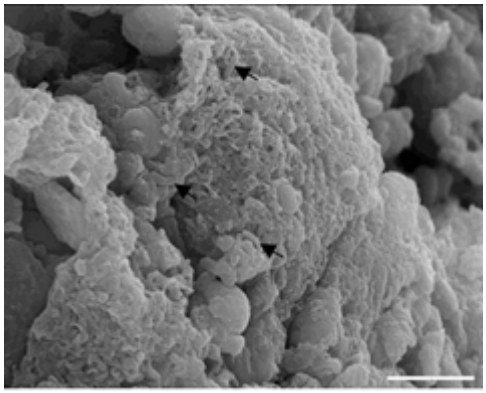

Figure 1: High magnification scanning electron micrograph of the jejunum of an inoculated rabbit (5 DPI). Bacteria (arrows) and mucus cover the tips of the villi. Bar = 10 µm

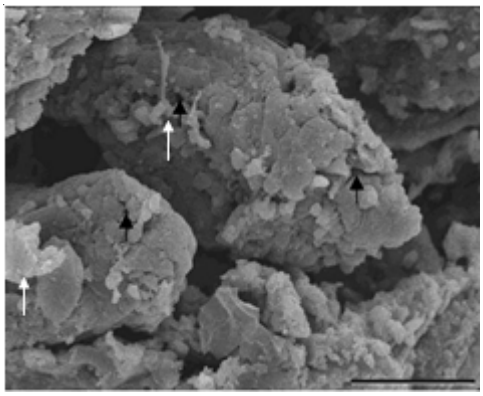

Figure 2: Scanning electron micrograph of the ileum of an inoculated rabbit. The tips of the villi are covered with mucus (white arrows) containing cellular debris. Epithelial cells are swollen and the epithelial integrity is disrupted (dark arrows). Bar = 50 µm.

These clinical signs were first observed at 3 DPI. At necropsy, the contents of the stomach and small intestine were very liquid. The caecal contents were either liquid or impacted. Congestion and inflammation in the duodenum, jejunum and ileum were observed in only 1 rabbit, slaughtered at 5 DPI. No clinical signs or gross lesions were observed in uninoculated animals. Apart from the changes reported in the gastrointestinal tract there was no evidence of any gross lesion elsewhere in the carcasses. No pneumonia was observed in animals presenting a large bowel impaction.

Ultrastructural findings and histopathological findings

SEM analysis of ileal and jejunal surfaces of additional infected rabbits demonstrated blankets and globular particles of mucus, reflecting goblet cell activation associated with numerous bacteria in 12 out of 15 rabbits. These lesions were observed in either the jejunal or the ileal sections and sometimes in both (Figures 1 and 2). No lesion was observed in control animals (Figure 3). In the rabbit showing the inflammatory lesions in the intestine at the necropsy, SEM examination demonstrated extensive lesions of the ileum and jejunum epithelium. These lesions were either mild or lacking in the other parts. The affected epithelia were packed with a mucus layer containing cellular debris; the tips of the villi were severely damaged while epithelial cells were swollen.

No consistent lesions were observed in the infected rabbits that presented only an aqueous intestinal content or in the control animals. TEM observations realized on selected cases of the jejunum or

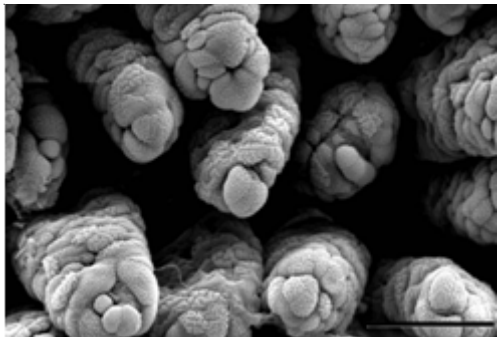

Figure 3: Scanning electron micrograph of the tips of the villi of the ileum of a control rabbit. Bar = 50 µm.

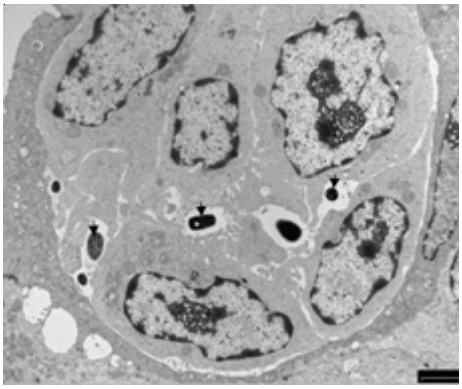

Figure 4: Transmission electron micrograph of a tangentional section near the bottom of an epithelial crypt in the ileum of an inoculated rabbit at 5 DPI. Bacteria are present in the intercellular space (arrows) Bar = 5 µm.

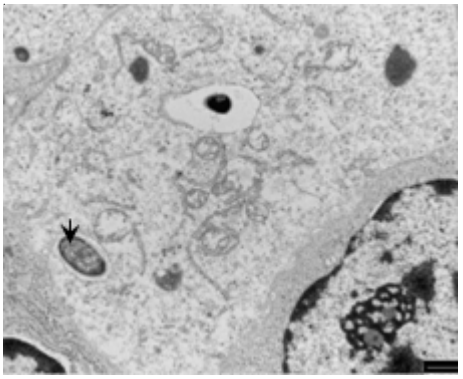

Figure 5: Transmission electron micrograph of the ileum of an inoculated rabbit at 5 DPI demonstrating bacteria in intracytoplasmic vacuoles. Bar = 2 µm

ileum showed the presence of bacteria in intracytoplasmic vacuoles or in the intercellular spaces in 3 out of 15 rabbits (Figures 4 and 5).

Histopathological findings are summarized in Table 2. The cellular infiltration in the *lamina propria* and the *submucosa* of the different parts of the intestine consisted mostly of polymorphonuclear leukocytes (PMN) associated with lymphocytes, macrophages and occasionally plasma cells. PMN's were more numerous in the lamina propria of inoculated animals compared to control animals. PMN infiltration in the *tunica muscularis* of the intestinal wall was observed in 6 out of 30 inoculated rabbits (Figure 6). These inflammatory cells were also observed near Auerbach's plexus (Figure 7).

The intestinal wall thickness (duodenum, jejunum, ileum or all three parts) was reduced in 8 out of 30 inoculated animals. Sometimes, a complete atrophy of the villi was observed (Figure 8). Major villous changes were fusion, destruction and loss of epithelial layers. Apoptosis of enterocytes and nuclear

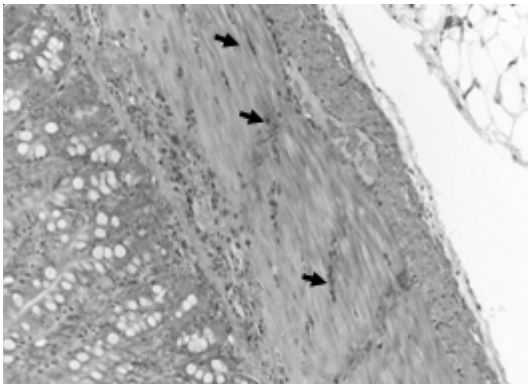

Figure 6: Proximal ileum of an inoculated animal (7 days post-infection) showing infiltration of PMN's (arrow) in the *tunica muscularis*. HE. Magnification 200×.

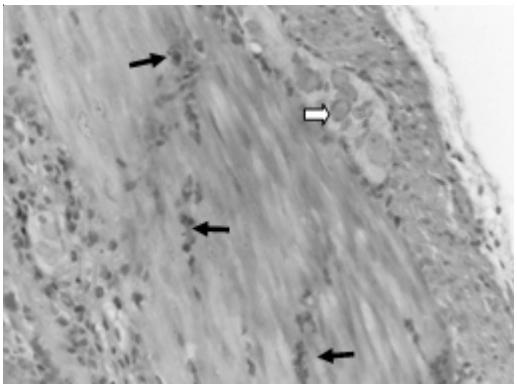

Figure 7: Proximal ileum of an inoculated animal (7 days post-infection) showing infiltration of PMN's (black arrow) in the *tunica muscularis* and evidence of degeneration of the myenteric plexus (white arrow). HE. Magnification 400×

Table 2: Histopathological findings in different intestinal areas: number of affected animals on the total number of inoculated or controls animals

Animals	Areas (total no. of animals)	Score	Lamina propia		Apoptotic enterocytes	Submucosa		Lumen		
			Dilation and congestion of blood vessels	Inflammatory cells infiltration (PMN)		Inflammatory cells infiltration	Dilation and congestion of blood vessels	Bacteria	Cellular debris and apoptotic cells	Food debris
Inoculated	Duodenum (n = 30)	0	3		1	3		16		
		1	18	15	16	20	12	6	11	15
		2	9	15	11	6	12	8	12	9
		3			2	1	6		7	6
	Jejunum (n = 30)	0		15	1				1	8
		1	4	15	22	3		14	17	8
		2	21			22	15	10	10	11
		3	5		7	5	15	6	2	3
	Ileum (n = 30)	0			3					6
		1	12	14	17	5	3	10	13	7
		2	11	15	8	15	11	11	9	7
		3	7	1	2	10	16	9	8	10
Controls	Duodenum (n = 30)	0	1		5			2		
		1	2	5		3	1	1	3	1
		2	2			2	4	2	2	3
		3								1
	Jejunum (n = 30)	0								1
		1	3	5	5		1	1	4	1
		2	2			1	4	2	1	3
		3				4		2		
	Ileum (n = 30)	0			3				1	1
		1	4	5	2	4		2	4	1
		2	1			1	3	2		3
		3					2	1		

Scores: 0, absence or normal; 1, mild; 2 moderate; 3, severe as described in materials and method

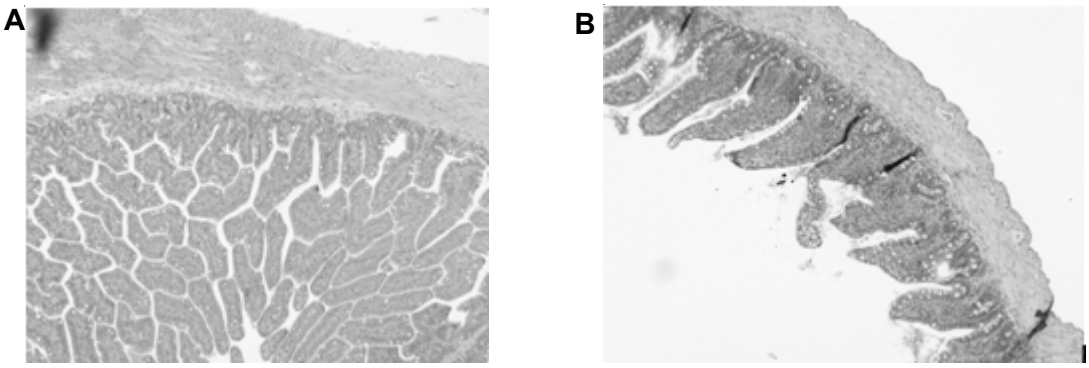

Figure 8: Histological examination of the proximal ileum of a non-inoculated rabbit (A) and of an inoculated rabbit, 3 days post infection (B). HE. Magnification 100×. The villi of the ileum in the diseased rabbit appear atrophied with irregular shape and size compared to those of the healthy animal.

debris were observed in the basal part of the villi and in the crypts. In the crypts apoptosis was observed in the duodenal sections from 6 out of 30 inoculated, but in none of the 5 control animals. It was also observed in the jejunal sections of 14 out of 30 inoculated and 1 out of 5 control animals; as well as in the ileal sections of 22 out of 30 inoculated animals and none of 5 control animals. There were more ($P<0.05$) apoptotic cells in the jejunal epithelium of inoculated rabbits than in control animals (Figure 9). In some cases, there were karyorrhexis and karyolysis of cell nucleus with vacuolization of the cellular cytoplasm and evidence of small-undefined intravacuolar particles.

Bacteria, detached cells and feed debris were found in the lumen of the three segments (jejunum, ileum, duodenum), in the inoculated as well as in control animals.

The scores for dilation and congestion of blood vessels of control animals varied between 1 and 2. In inoculated rabbits, the mean scores (≥ 3) for dilation and congestion of blood vessels of the jejunal *lamina propria* were significantly higher ($P<0.005$) than in control rabbits. This was not the case in the *lamina propria* or in the *submucosa* of the other parts of the digestive tract.

The others organs (liver, lung, spleen, stomach, and heart) were not examined because any changes were observed in previous studies (Marlier *et al*., 2005; Licois *et al*., 2005). Warthin-Starry staining showed adhesion of bacilli on the villi and crypts of the ileum and jejunum in one inoculated animal (Figures 10 and 11). In another rabbit, bacilli invading the dome epithelial cells were observed.

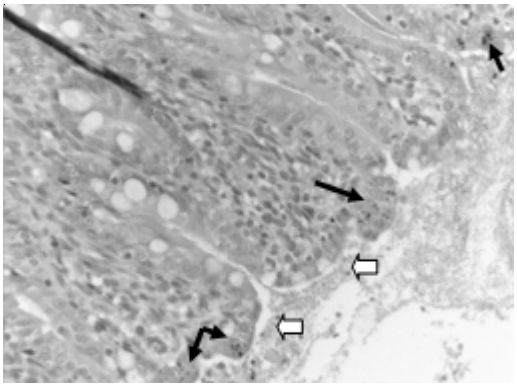

Figure 9: Distal ileum of an inoculated animal (5 days post-infection) showing apoptosis of the enterocytes (black arrow) and necrotic debris (white arrow). HE. Magnification 400×.

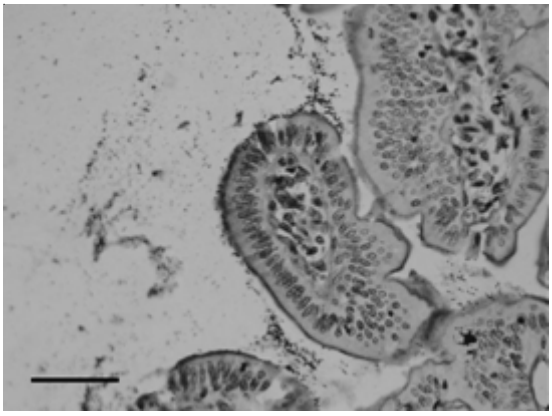

Figure 10: Light microscopy of the jejunum of an inoculated rabbit showing bacteria adhering to villi. Warthin Starry stain (Bar = 25 µm)

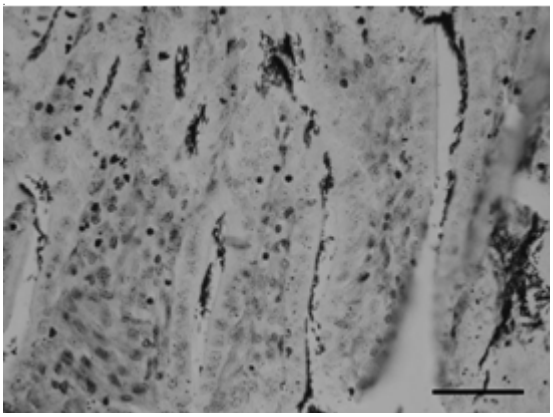

Figure 11: Light microscopy of the ileum of an inoculated rabbit where bacteria adhering to crypts are present. Warthin Starry stain (Bar = 25 µm)

Bacteriological examinations

The bacterial strains isolated from mixed intestinal contents of inoculated rabbits were, in aerobic conditions: non-enteropathogenic *Escherichia coli*, *Bacillus* spp, group D streptococci and, in anaerobic conditions: *Clostridium perfringens* and *Fusobacterium necrogenes* (Table 3). All *Escherichia coli* strains isolated from inoculated animals did not agglutinate with antisera against O antigens (O 15, O 109, O 103, O 128 and O 132). The *eaeA* gene was never detected by PCR amplification.

Haematological findings

There was no modification of erythrocyte, haemoglobin or heamatocrit count, in the inoculated compared to control animals, before and after inoculation. No significant difference of total leukocyte counts was observed between the two groups before and after inoculation (Table 4). However, after inoculation, the neutrophil and lymphocyte counts of the two groups were significantly different ($P<0.05$ and $P<0.01$, respectively), with neutrophil rate being increased and leukocyte rate being decreased, significantly compared to those of non-inoculated control rabbits (Figure 12).

DISCUSSION

In the field and in absence of antibotherapy, ERE is an acute disease with a high mortality rate (Licois *et al*., 2005). In this study, no spontaneous mortality appeared. It has already been mentioned, in experimental cases of ERE, that mortality strongly varies (Licois *et al*., 2005) and is thus not a highly reliable criterion. Another assumption could be that the animals were killed before dying

Table 3: Bacteria isolated from mixed small intestinal contents (jejunum, ileum) of 15 inoculated rabbits and two controls animals in aerobic and anaerobic condition

	Inoculated (n= 15)															Control (n=2)	
Rabbits number	2	3	4	5	8	9	11	13	14	15	17	20	23	27	29	1	2
E. coli	+	+	0	0	+	0	+	+	+	+	+	+	+	+	+	0	0
Bacillus spp	+	0	+	+	+	+	0	+	+	+	+	0	+	+	+	+	+
Group D streptococci	0	0	0	+	0	+	+	+	0	0	+	0	+	0	+	+	0
Cl. perfringens	+	0	0	+	0	0	0	0	+	+	0	0	0	0	0	0	0
Fusobacterium necrogenes	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0

+, presence; 0, absence.

Table 4: Mean ± standard deviation of haematological profile (values×10^9/l) in control and inoculated rabbits

Blood parameters	Before inoculation			After inoculation		
	Inoculated (n=17)	Control (n=5)	Significance	Inoculated (n=17)	Control (n=5)	Significance
Leucocytes	4.22±1.10	3.34±0.27	NS	5.88±2.83	4.29±1.40	NS
Neutrophils	1.04±0.43	0.83±0.25	NS	4.10±2.55	1.54±0.69	$P<0.05$
Lymphocytes	2.67±0.66	2.12±0.41	NS	1.04±0.57	2.20±0.98	$P<0.01$
Monocytes	0.42±0.17	0.30±0.16	NS	0.63±0.41	0.40±0.10	NS
Eosinophils	0.007±0.008	0.002±0.004	NS	0.004±0.006	0.002± 0.004	NS
Basophils	0.13±0.07	0.08±0.05	NS	0.11±0.12	0.19±0.19	NS

spontaneously. However, we were able to reproduce clinical signs and gross lesions typical of ERE, described previously by other scientific workers such as abdominal bloating, cecal impaction, diarrhoea and mucus secretion (Licois *et al*., 1998; Licois *et al*.,2005).

Clinical signs and macroscopical lesions of ERE have some similarities with those of mucoid enteritis (ME) first described by Muir (1943) and re-discovered later by Van Kruiningen and Williams (1972). However, ME cases which were reported in England and in the United States, were mainly sporadic outbreaks whereas ERE is an epidemic disease. The results of this study show that ERE as well as ME (Van Kruiningen and Williams, 1972) can be reproduced by oral inoculation but there are too many unknown factors for both diseases to establish any putative link. In the present study, the clinical signs and the macroscopical lesions observed seem more severe at the beginning of the observation period than at the end. The fluid accumulation in the stomach and the small intestine and the paucity of the inflammatory response strongly suggest secretory diarrhoea. Some bacterial toxins such as enterotoxins can contribute to stimulate active chloride secretion and/or inhibits electroneutral NaCl absorption in the intestinal epithelium. This mechanism does not lead to histological modification (Sears and Kaper, 1996).

The microscopic observations demonstrate the limited extent of the inflammatory lesions. However, the vascular changes (dilation and congestion) were mostly observed in the jejunal *lamina propria* and submucosal vessels of inoculated rabbits. In some cases, the nuclear debris observed without inflammatory cell infiltration might be linked to programmed cell death or post-mortal changes. The most noticeable lesion was the apoptosis of cells of jejunal epithelium and crypts and the presence of nuclear debris. All samples of intestinal tract were taken immediately after euthanizing rabbits and fixed immediately in formalin; but, some artefacts cannot be totally excluded. Sometimes the intestinal

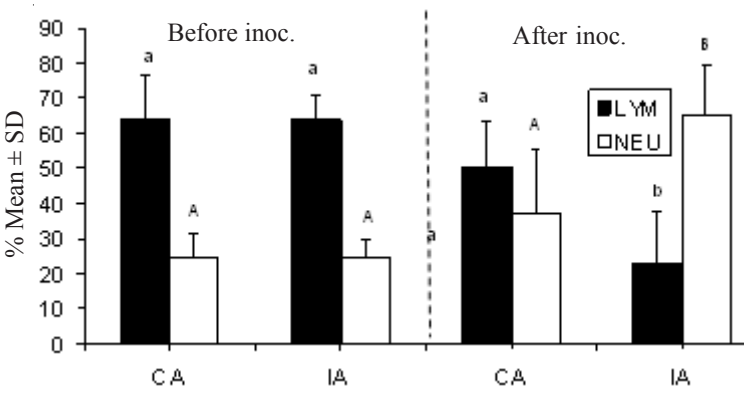

Figure 12: Mean of percentage of neutrophils (NEU) and lymphocytes (LYM) and standard deviation in the control (CA) and inoculated animals (IA), before and after inoculation. Mean values with common superscripts are not significantly different ($P<0.05$)**.**

mucosa was characterized by villous atrophy, fusion or destruction and loss of the epithelial layers of villi. This again points to loss of cells by apoptosis. It is difficult to interpret fully these results because the microscopic lesions of the small intestine were not constant and sometimes limited to a small part of the intestinal tissue or, were diffused in all intestinal sections. The infiltration of PMN observed into the jejunal or ileal *muscularis* can affect the intestinal mobility especially with PMN being present near Auerbach' plexus (Eskandari *et al*., 1997; Hierholzer *et al*., 2001).

The bacteria observed in the epithelium by transmission electron microscopy had a similar morphology. They were short rods with an undulating outer membrane about 1500 nm long and 700 nm in wide. The morphology of these bacteria in TEM was consistent with the description of gram-negative bacteria, which possess an undulating outer membrane (Cheville, 1994). Sometimes the size of rods seemed different what can be easily interpreted according to the cutting direction. In some animals, bacteria with bacilli morphology were observed attached at site of serious cell damage. These bacteria appeared only in the ileum of animals inside the epithelial cells or in the intercellular space. These findings confirm the results obtained with Warthin-Starry staining. The role of these bacteria remains unclear because they were detected in a few animals only. They may either play a role in ERE or may be considered as secondary invaders.

The bacteriological examinations led only to the isolation of typical non-pathogenic or opportunistic bacterial strains. Only the presence of *C. perfringens* strains can be debatable because this bacterium is sometimes found in faeces of healthy rabbits (Lee *et al.*, 1991) but also in enteric disease cases (Songer, 1996). The same observations have been made in a previous study (Marlier *et al*., 2005).

The neutrophilia is pathologically induced by infections whereas lymphopenia occurs with acute severe disease, some viral disease or is stress-related (Sodikoff, 2001). The increase in the blood neutrophils to lymphocytes ratio is a possible marker of a stress response (Merlot, 2004) but also might reflect systemic inflammation (Zahorec, 2001). An increase of neutrophyl polynuclear has already been observed in experimentally induced ERE by Jobert *et al*. (2001). Normally, the percentage of lymphocytes is higher than neutrophils in this specie (Aleman *et al.*, 2000) whereas in our study an inversed trend was noted. The neutrophilia and lymphopenia observed in inoculated animals could be a response of the immune system to inflammation and this could reinforce the hypothesis of an infectious cause such as bacterial invasion. The same conclusion about a bacterial aetiology in the ERE syndrome has recently been mentioned by Szalo *et al*. (2006).

In conclusion, the aetiopathogenesis of ERE cannot be determined from this study. However, several histological and ultrastructural lesions and haematological profiles observed in this present study at the same time corroborate an infectious aetiology and suggest a bacterial origin as mentioned by Szalo *et al*. (2006). It is possible that ERE is a multifactorial disease. It is unclear whether the intra or intercellular bacteria, which were observed in the ileal epithelium of some animals, are primary or secondary invaders involved in the pathogenesis of this syndrome. Further investigations will need to be undertaken in order to show the putative role of bacteria in ERE.

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