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Infectious agents associated with epizootic rabbit enteropathy: Isolation and attempts to reproduce the syndrome

D. Marlier [a,*], R. Dewrée [a], C. Lassence [a], D. Licois [b], J. Mainil [c], P. Coudert [b], L. Meulemans [d], R. Ducatelle [d], H. Vindevogel [a]

[a] *University of Liège, Faculty of Veterinary Medicine, Department of Infectious and Parasitic Diseases, Birds and Rabbits Medicine, Sart Tilman, Bât B42, B4000 Liège, Belgium*
[b] *INRA, BioAgresseurs, Santé, Environnement, 37380 Nouzilly, France*
[c] *University of Liège, Faculty of Veterinary Medicine, Department of Infectious and Parasitic Diseases, Bacteriology, Sart Tilman, Bât B43, B4000 Liège, Belgium*
[d] *University of Ghent, Faculty of Veterinary Medicine, Department of Bacteriology, Pathology and Avian Diseases, Salisburylaan, 133, B9820 Merelbeke, Belgium*

Abstract

Epizootic rabbit enteropathy (ERE), a highly lethal (30–80% mortality) disease of broiler rabbits aged 6–14 weeks, first appeared in 1997 in French intensive enclosed rabbitries and is of unknown aetiology. Bacteriological, virological and parasitical examination of the intestinal contents of rabbits that had died either in spontaneous field cases or after experimental reproduction of ERE, were undertaken in an attempt to identify infectious agents that may play a role in the disease. Two bacterial strains, *Clostridium perfringens* and non-enteropathogenic *Escherichia coli*, were repeatedly isolated at high faecal counts from naturally infected animals. In field cases, a correlation between typical gross lesions of epizootic enteropathy and the presence of the alpha toxin of *Cl. perfringens* was observed ($P < 0.0001$; Chi-squared test). Although attempts to reproduce the disease by inoculation with different pools of cultivable bacterial strains failed, the disease was successfully reproduced by inoculation with one French and two Belgian samples of caecal contents.

Keywords: Epizootic rabbit enteropathy; *Oryctolagus cuniculus*; Aetiology; *Clostridium perfringens*; Inoculation

1. Introduction

Since it was first identified in 1997, Epizootic Rabbit Enteropathy (ERE) has become a serious concern for rabbit breeders in most European countries (Coudert et al., 1997; Lebas and Coudert, 1997) and causes significant economic losses in rabbit farms. The first outbreak was characterized by a sudden onset of abdominal distension and some diarrhoea in 6–14-week old rabbits (Licois et al., 1998). Gross lesions included dilatation of the stomach and the small intestine with mainly liquid contents. The caecum was either impacted or contained liquid, and mucus was occasionally present in the colon (Licois and Coudert, 1999). Subsequently, these pathological lesions systematically appeared in other batches of rabbits showing similar clinical signs of ERE. Definitive diagnosis can however be difficult, and although it is based on the observation of the characteristic lesions, these can be modified by supervening secondary bacterial infections (Licois et al., 1998; Marlier and Vindevogel, 1998).

There are reports of the transmission of ERE to weaned specific-pathogen-free (SPF) New Zealand White rabbits by pulverizing feed with the intestinal contents of sick animals (Licois et al., 1998), but despite

* Corresponding author. Tel.: +32 4 366 40 19; fax: +32 4 366 40 12.
E-mail address: dmarlier@ulg.ac.be (D. Marlier).

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several toxicological, bacteriological, parasitological and virological examinations, the aetiology of ERE remains unknown. (Lebas, 1998; Licois and Coudert, 1999; Licois et al., 1999).

The results of our bacteriological, virological and parasitical study of naturally infected rabbits are presented in the first part of this report. The second part is devoted to experimental reproduction of the disease by administration of two Belgium samples, which are compared with a French one. In the third part, the results of our attempt to reproduce the disease by inoculation of bacterial isolates extracted from the intestinal contents of sick animals are described.

2. Material and methods

2.1. Microbiological characterisation of faecal contents from rabbits with ERE and toxin detection (field cases)

Two groups of rabbits originating from eight rabbitries affected by intestinal pathology were studied. In the first group, 28 animals, which had died from ERE, were submitted for a complete post mortem examination. Death rates and ages of affected animals on each farm were recorded. Eighteen animals were typical ERE cases, with no obvious supervening secondary infection signs. The contents of the stomach, small intestine and caecum of 12 animals were collected for bacteriological, virological and parasitical examination. In addition, 62 isolates of *Clostridium perfringens*, isolated from the intestinal contents of six animals (10–12 strains/rabbit) that had died from ERE were typed.

In the second group, 86 rabbits, which had died with digestive pathology, were used for the detection of clostridial toxins (alpha, beta and enterotoxin of *Cl. perfringens*, toxins A and B of *Cl. difficile*) in the digestive contents. There was a clear clinical evidence of ERE in 32 rabbits and 54 rabbits had signs of other intestinal pathologies.

2.2. Parasitical examinations

Caecal contents were examined for coccidial oocysts and for helminth eggs by the salt-flotation-concentration technique (Troncy et al., 1981) and the number of oocysts and eggs was recorded after counting in a McMaster cell. The caecal contents were also submitted for direct examination to corroborate the salt-flotation technique.

2.3. Virological examinations

The presence of rotavirus in caecal contents was investigated using a commercial ELISA Kit for bovine rotaviruses (Cypress Diagnostics). The suitability of this Kit for rabbit rotavirus detection was tested with known rabbit rotavirus-infected intestinal contents.

2.4. Bacteriological examinations

For the qualitative examinations, samples were serially diluted 10-fold in sterile physiological saline solution (0.9% NaCl) and 100 μL of each dilution were inoculated onto appropriate media. The plates were incubated aerobically at 37 °C for 24 h or in an anaerobic chamber at 37 °C for 24–48 h (MK3 Anaerobic Work Station, Led-Techno). The media used in the aerobic conditions were 5% sheep blood agar, Columbia-ANC agar, MacConkey agar, Schiemann Cin agar, TCBS Cholera agar, Salmonella–Shigella agar. For anaerobic conditions, we used Schaedler agar and TSN agar (Oxoid).

For the isolation of *Campylobacter* spp., samples were plated on Buttzler and Karmali agar (microaerophilia, 42 °C, 48 h) (Oxoid). Bacterial colonies were identified according to standard procedures (Barrow and Feltham, 1993). Isolates were further identified by API 20 Strep, Api 20 Staph, Api 20 NE, Api 20 E, Api 50 CH and Api ID 32A (BioMérieux) according to the manufacturer's instructions. All *E. coli* isolates were biotyped and serotyped according to the recommendations of Peeters et al. (1988) and Okerman (1998).

Some *E. coli* isolates were also tested for the presence of the *eae*A gene by PCR as described by China et al. (1996). The reference strain *E. coli* RDEC-1 (O15:H-) was used as a positive control. The primer sequences used and references are given in Table 1. DNA extractions were performed as described by Meer and Songer (1997). PCR reactions were performed in a DNA thermal cycler, Minicycler (MJ Research Biozym). Electrophoretic migrations were carried out through 1% agarose gel (1 h at 100 V). To complete the bacteriological examinations, the caecal contents were also Gram stained after centrifugation (20,000*g*, 4 °C, 15 min) before being submitted for *Cl. spiroforme* detection as recommended by Holmes et al. (1988), and subjected to Vago staining for spirochetes (Vago, 1953).

Some additional quantitative bacteriological examinations for *Cl. perfringens* and *E. coli* counts were performed on 12 selected intestinal contents by plating serial 10-fold dilutions of intestinal contents onto Schaedler agar and MacConkey agar (Oxoid) and incubating them under anaerobic and aerobic conditions.

To determine more accurately the type of *Cl. perfringens* found in ERE cases, 10–12 stains isolated from one single animal per rabbitry and four strains from a control animal were toxinotyped (for major toxins) by PCR. The presence of beta$_2$ toxin was detected by the method of Herholz et al. (l999). The reference strains of *Cl. perfringens* (CN 3888, CWC 236, NCIB 10748, CN 3978, 46960-C8) were used as positive controls.

Table 1
Sequences of primers and PCR conditions for *Cl. perfringens* toxinotyping and *E. coli eae*A gene amplification

Gene	Primer sequences 5′-3′	Optimal annealing temperature (°C)	Product size (bp)	Reference
Cpa	TGCTAATGTTACTGCCGTTGATAGATAATCCCAATCATCCCA-ACTATG	51.7	247	Titball et al. (1989)
Cpb1	GCGAATATGCTGAATCATCTAGCAGGAACATTAGTATATCTTC	53	196	Meer and Songer (1997)
Ipa et ipb	TTTTAACTAGTTCATTTCCTAGTTATTTTTGTATTCTTTTTCTC-TAGGATT	46.2	298	Daube et al. (1994)
Cpb2	GAAAGGTAATGGAGAATTATCTTAATGCGCAGAATCAGGATT-TTGACCATATAC	48	573	Herholz et al. (1999)
Etx	GCGGTGATATCCATCTATTCCCACTTACTTGTCCTACTAAC	53	655	Meer and Songer (1997)
*eae*A	AGGCTTCGTCACAGTTGCCATCGTCACCAGAGGA	51.9	570	China et al. (1996)

The primer sequences and PCR conditions used and references for *Cl. perfringens* major toxins genes are given in Table 1. DNA extraction techniques and electrophoretic migration were the same as those described for *E. coli eae*A gene amplification.

2.5. Detection of toxins

The presence of *Cl. perfringens* alpha toxin, beta toxin and enterotoxin in intestinal contents of the 86 animals that had died of ERE ($n = 32$) or of other intestinal disorders ($n = 54$) was investigated using commercial Kits. (Alpha toxin and Beta toxin: ELISA Kit, Cypress diagnosis; *Clostridium perfringens* enterotoxin: Enterotoxin test, Techlab; *Cl. difficile* Tox A/B II, Techlab.). The results were analysed for significance by the Chi-squared test. *P* values of 0.05 were considered significant.

2.6. Experimental reproduction of ERE with different inocula

Inocula Belgium B1 and B2 were obtained during 2002 from diseased rabbits originating from two rabbit farms in which typical ERE clinical signs repeatedly occurred in growing rabbits. Both inocula were prepared as described by Licois and Coudert (2001). Caecal contents from two rabbits that died at six (B1 inoculum) weeks of age or from one rabbit that died at seven weeks of age (B2 inoculum) were diluted at 1:3 in sterile water, grossly filtered at 0.5 mm and centrifuged at 1000*g* for 15 min. The supernatant was used as inoculum. This treatment did not remove viruses or bacteria but it eliminated coccidia (Licois and Coudert, 2001). The reference French inoculum (TEC3) described by Licois and Coudert (2001) was also used as a control. Before use, the inocula were submitted to bacteriological, virological and parasitical examinations as previously described.

One hundred and seventy-four seven-week-old SPF New Zealand White rabbits (Unité Expérimentale de Pathologie Aviaire et de Parasitologie, INRA, Nouzilly, France) were used. Breeding conditions and experimental facilities have been outlined previously (Licois and Coudert, 2001).

All care and experimental procedures involving animals followed the guidelines stated in the *Guide for the Care and Use of Laboratory Animals* (1996).

The first experiment was performed using three groups housed in different rooms. Thirty-six rabbits were each inoculated with 1.3 mL of inoculum B1 each; 24 animals were each inoculated with 500 μL of TEC 3 and 27 were kept as control animals, each receiving 500 μL of intestinal contents obtained from healthy SPF rabbits.

In the second experiment, 38 animals were each given 500 μL of inoculum B2 and 25 received 500 μL of TEC 3. Twenty-four animals were kept as controls and inoculated as described above. In both cases the three groups were kept in separate rooms. All test substances were administered by the oral route.

After administration, the animals were observed twice a day and weighed three times a week over a two-week period. The mean daily weight gains were analysed by the Kruskal–Wallis non-parametric ANOVA test and Dunn's multiple comparisons test. A *P* value of 0.05 was considered significant.

2.7. Experimental reproduction of ERE with isolated bacteria

Seventy-six seven-week-old SPF New Zealand White rabbits were used (INRA, France). The animals were divided into four groups (three groups of 20 rabbits each, and one control group of 18 rabbits). After administration of the suspensions, the animals were examined daily and weighed once a day over a 10-day period.

Enterococcus faecium, Enterococcus faecalis, *Bacillus licheniformis*, *Bacillus sphaericus* and *Bacillus pumilus* bacterial strains were plated onto sheep blood agar under aerobic conditions and grown for 24–48 h. *Cl. perfringens* and *Cl. sordellii* bacterial strains were plated onto Schaedler agar under anaerobic conditions and grown for 24–48 h. From these bacterial cultures, three suspensions composed of pooled bacterial strains

diluted with physiological salt solution were prepared. The first included approximately 10^7 colony forming unit (cfu) per millilitre of *Cl. sordellii* (six different strains) and 10^6 cfu/mL *Cl. perfringens* (two strains); the second (a pool of all the TEC3 cultivable bacterial species) had approximately 10^7 cfu/mL *Enterococcus faecium* and *Ent. faecalis*, 10^6 cfu/mL *Bacillus licheniformis*, 10^6 cfu/mL *Bacillus pumilus*, 10^7 cfu/mL *Bacillus sphaericus*, 10^7 cfu/mL *Cl. sordellii* and 10^6 cfu/mL *Cl. perfringens*. The third suspension was made up of approximately 10^7 cfu/mL *Bacillus licheniformis* (four strains). Each rabbit received 500 μL/mL of the different suspensions. Since ERE is fully reproduced by TEC3 inoculum, which is free of any *E. coli* (Licois et al., 2005), no *E. coli* strains were included in the inocula.

2.8. Histopathological examinations

Fifty-six seven-week-old SPF New Zealand White rabbits were used (Charles River laboratories Belgium). Ten rabbits were each inoculated with 0.5 mL of inoculum TEC2 (Licois et al., 2005) and five were kept as control animals. Three animals (one control and two inoculated) were each killed at 1, 2, 4, 5 and 8 days post infection (DPI), the abdomens were opened and samples from lung, liver, stomach, jejunum, ileum, caecum and proximal colon were collected and fixed in 10% neutral buffered formalin. The tissue specimens were embedded in paraffin wax, cut at 5 μm and stained with haematoxylin and eosin (HE). Sections were examined under light microscopy.

3. Results

3.1. Study of the field cases

The histories, ages of affected animals and number of rabbits with ERE/number of necropsies are given in Table 2. Noticeable dilatations of the whole digestive tract (including the stomach) with liquid contents but without signs of inflammation were observed in 18/28 rabbits. Sometimes the lesions were linked to caecal paresis. An acute congestion of the small intestine was found in all animals considered non-typical ERE cases.

The bacterial genus and/or species isolated in aerobic or anaerobic conditions are presented in Table 3. In qualitative studies, *E. coli* and *Cl. perfringens* were the most frequently encountered bacterial species. Both biotyping/serotyping and PCR reactions failed to demonstrate any enteropathogenic *E. coli* strains. The *E. coli* and *Cl. perfringens* faecal counts were above 10^4 cfu per gram of faeces (cfu/gf), respectively, in 66% and 83% of the cases. The *Eimeria* spp. oocysts counts were above 5000 oocysts per gram faeces in 40% of cases but no gross coccidiosis lesions were detected at necropsies. No *Cryptosporidium* oocysts were observed. Finally, the presence of rotavirus in faeces was found in 33% of cases.

Table 3
Results of bacteriological examinations in aerobic and anaerobic conditions of mixed faecal samples (stomach, small intestine and cæcum) of 12 rabbits that had died of ERE (field samples)

Bacterial strain	Number of positive examinations/total number of examinations
Enteropathogenic *E. coli*[a]	0/12
E. coli (>10^4 FCU/gf)	8/12
E. coli (<10^4 UFC/gf)	3/12
No *E. coli*	1/12
Enterococcus casseliflavus and/or *durans*	3/12
Staphylococcus spp.	3/12
Streptococcus spp.	1/12
Bacillus licheniformis	1/12
Bacillus sphaericus	2/12
Clostridium perfringens	10/12
Clostridium paraputrificum	1/12
Clostridium ramosum	1/12
Clostridium fallax	1/12
Clostridium histolyticum	2/12
Clostridium baratii	2/12
Capnocytophaga spp.	1/12
Bacteroïdes spp.	3/12

[a] Both bio-serotyping and PCR amplification for the *eae*A gene were negative.

Table 2
Histories, ages of affected animals and number of rabbits with ERE/number of necropsies in the eight rabbit farms

Rabbitries	History	Age of the animals (weeks)	Number of rabbits with ERE/number of necropsies
1	Mortality rate >50%	6	3/3
2	Digestive outbreaks just after the weaning	6	1/1
3	Mortality rate > 50%	7	3/6
4	Mortality rate > 50%	6–11	4/6
5	Mortality rate > 50%	6–13	2/7
6	Mortality rate < 50%	9	1/1
7	Digestive outbreaks just after the weaning	8	1/1
8	Digestive outbreaks just after the weaning	8	3/3

Only major *Cl. perfringens* toxin types A and C were found. In three farms all the strains belonged to toxin type A, in one farm all the strains were of type C and in two farms mixed types A and C were found. Only one isolate was found to be positive for the B_2 gene. In control animals only major toxin type A was found.

3.2. Toxin detection

The presence of *Cl. perfringens* alpha toxin was detected in 31 animals; nine rabbits died of non-ERE digestive disorders and 22 died of ERE. However, alpha toxin was not however detected in the intestinal contents of 55 animals among which 54 had died of non-ERE disorders ($P < 0.0001$; Chi-squared test). *Cl. perfringens* beta toxin and enterotoxin, and *Cl. difficile* A and B toxins were not detected in faecal contents.

3.3. Experimental reproduction of the ERE

The bacterial strains isolated from TEC3 under aerobic conditions were *Bacillus pumilus* (10^3 cfu/gf), *Bacillus licheniformis* (10^4 cfu/gf), *Bacillus sphaericus* (10^5 cfu/gf), type D *Streptococcus* such as *Enterococcus faecium* and *faecalis* (10^5 cfu/gf). Under anaerobic conditions, *Cl. sordelii* (10^5 cfu/gf), *Cl. butyricum* (10^5 cfu/gf) and *Cl. perfringens* (10^6 cfu/gf) were found. The inoculum was free of *E. coli*, *Salmonella* spp., *Yersinia* spp., *Campylobacter* spp. and *Vibrio* spp. A very limited amount of *Cl. spiroforme* was observed and only when an enrichment centrifugation step was performed before the microscopic examination. The inoculum was free of *Eimeria* spp. but a positive reaction was found with rotavirus ELISA test. The same bacterial strains were found in both Belgium B1 and B2 inocula. The most frequent bacterial species were non-enteropathogenic *E. coli* and *Cl. perfringens* with counts $>10^4$ cfu/gf. The ELISA rotavirus tests were positive for both inocula. *Eimeria* species were found in both cases but a count of >5 000 oocysts per gram of faeces was observed only for inoculum B2.

Table 4
Comparison of ERE clinical signs rates, mortality rates and mortality ranges after experimental reproduction of ERE in SPF rabbits inoculated with two Belgian and one French inoculum

Inoculum (number of rabbits)	ERE[c] clinical signs rate (%)	Mortality rate (%)	Mortality range in days
Controls 1[a] ($n = 27$)	0	0	–
B1[a] ($n = 36$)	33	22	4–11
TEC3[a] ($n = 24$)	54	33	4–13
Controls 2[b] ($n = 27$)	0	0	–
B2[b] ($n = 38$)	87	16	4–11
TEC3[a] ($n = 25$)	100	24	3–25

[a] Animals used in the B1 inoculation experimentation.
[b] Animals used in the B2 inoculation experimentation.
[c] Rambling noise and/or diarrhoea and/or stomach distension and/or intestinal distension were considered ERE clinical signs.

The results of inoculation with B1, B2 and TEC3 are shown in Tables 4 and 5. At necropsy of spontaneously dead animals, the gross lesions observed were intestinal dilatation with liquid content but without inflammatory lesions. Before inoculation, the DWG did not significantly differ between the groups. Throughout the experiment the DWG of the TEC3 inoculated groups significantly differed from those of the control group. For the B1 and B2 inoculated rabbits, the DWG significantly differed from the control groups from day 3 to day 10 after inoculation.

3.4. Inoculations with pooled bacterial strains

ERE was not reproduced following the inoculation of any of the three bacterial pools (10^7 cfu *Cl. sordellii* and 10^6 cfu *Cl. perfringens*/mL inoculum; 10^7 cfu *Ent. Faecium* and *faecalis*, 10^6 cfu *Bacillus licheniformis*, *Bacillus pumilus*, 10^7 cfu of *Bacillus sphaericus*, 10^7 cfu *Cl. sordellii* and 10^6 cfu *Cl. perfringens*/mL inoculum; 10^7 cfu *Bacillus licheniformis*/mL inoculum). No rabbit died in the first group, one animal died in group 2, two in group 3 and one in the control group. No ERE lesions were observed at necropsy of those animals. DWG of the four groups were 40, 39, 38 and 40 grams per day, respectively.

Table 5
Mean daily weight gains (mean ± standard deviation) during experimental reproduction of ERE in SPF rabbits inoculated with two Belgian (B1 and B2) and one French (Tec3) inoculum

Days after inoculation	Control group B1 experimentation	Rabbits inoculated with B1	Rabbits inoculated with TEC3	Control group B2 experimentation	Rabbits inoculated with B2	Rabbits inoculated with TEC3
0–3	43 ± 6.5 ($n = 27$)	$22 \pm 13^*$ ($n = 36$)	$8.3 \pm 18^*$ ($n = 21$)	34 ± 8.0 ($n = 24$)	$26 \pm 14^*$ ($n = 38$)	$16 \pm 9.6^*$ ($n = 24$)
3–6	46 ± 5.2 ($n = 27$)	$24 \pm 23^*$ ($n = 31$)	$19 \pm 17^*$ ($n = 17$)	45 ± 5.2 ($n = 24$)	$21 \pm 23^*$ ($n = 33$)	$9.2 \pm 16^*$ ($n = 22$)
6–10	45 ± 5.6 ($n = 27$)	$23 \pm 18^*$ ($n = 28$)	$30 \pm 17^*$ ($n = 14$)	45 ± 4.8 ($n = 24$)	$25 \pm 22^*$ ($n = 32$)	$24 \pm 23^*$ ($n = 20$)
10–13 (B1) 10–14 (B2)	43 ± 6.5 ($n = 27$)	39 ± 9.9 ($n = 28$)	$22 \pm 25^*$ ($n = 13$)	47 ± 8.7 ($n = 24$)	41 ± 10 ($n = 32$)	$32 \pm 14^*$ ($n = 19$)

n = number of data.
* Significantly different from the respective control group ($P < 0.05$) by the Kruskal–Wallis non-parametric ANOVA test and Dunn's multiple comparisons test.

3.5. Histological examination

None of the five control rabbits or the 15 inoculated animals presented any histological changes in the lung, liver, stomach, caecum and colon. At 4 ($n = 1$), 5 ($n = 1$) and 8 ($n = 1$) DPI, some mild infiltration of lymphocytes or eosinophilic polymorphonuclear were observed in the jejunum and ileum of inoculated rabbit. At 4 ($n = 2$) and 5 ($n = 1$) DPI, post mortem desquamation of villi epithelium and mild atrophy of villi were observed in inoculated animals.

4. Discussion

The bacteriological examinations performed on the digestive contents of ERE field cases led to the isolation only of typical non-pathogenic or opportunistic bacterial strains. The first characteristic of the intestinal bacterial flora of ERE-affected rabbits seems to be the limited number of cultivable bacterial species. Normally, strict anaerobic bacteria (*Bacteroides* spp.) and facultative anaerobic bacteria such as *Streptococcoci* (Licois, 1989; Okerman, 1998) dominate the caecal flora of rabbits. In this study, there were few rabbits from which *Bacteroides* spp. were successfully isolated from caecal contents whereas this bacterium would normally be expected to be present at a high level (10^8–10^9 bacteria per gram) in almost all rabbits (Gouet and Fonty, 1979).

Additionally, no rabbit-specific pathogenic bacterial isolate was found in the different inocula used, with the exception of a slight contamination of TEC3 by *Cl. spiroforme*. This bacterium is an anaerobic Gram-positive microorganism that may cause an enterotoxaemia-like disease linked to the production of a lethal enterotoxin antigenically closed to the *Cl. perfringens* iota toxin (Holmes et al., 1988). The asymptomatic carriage of this bacterium in rabbits is quite common (Borriello and Carman, 1983). However, the typical gross lesions (Baker, 1998) seen in *Cl. spiroforme* enterotoxaemia (a light to severe acute haemorrhagic necrotic typhlitis) are very different from those observed in ERE.

In 66% of the ERE field cases, *E. coli* faecal counts above the physiological limit of 10^4 cfu/gf (Padilha et al., 1996) were observed. It may be surprising that only limited attention was paid to *E. coli* infection in this study. Indeed, the demonstration from the start: (1) that the TEC3 inoculum was totally *E. coli*-free and, (2) that the ERE was fully reproduced by inoculation with TEC3 demonstrated that *E. coli* was not the aetiological agent of ERE (Licois et al., 2005). So analysis conducted on *E. coli* strains were done specifically to avoid misdiagnosis. In rabbits, epizootics of colibacillosis are characterized by infection with attaching-effacing *E. coli* strains (for a review see Wales et al., 2005) that rarely produce enterotoxins or verotoxins (Pohl et al., 1993; Blanco et al., 1997). In rabbits with intestinal colibacillosis, the presence of the *eae* gene is closely associated with diarrhoeal disease (Blanco et al., 1997).

Also, high *Cl. perfringens* faecal counts were detected in the digestive contents of 80% of the ERE field cases. This bacterial species is sometimes found in healthy animals including rabbits (Lee et al., 1991) but is usually considered one of the most widely occurring pathogenic bacteria and the first cause of clostridial enteric disease in animals (Songer, 1996). *Cl. perfringens* strains can be classified into five toxinotypes A to E on the basis of the production of one or more of the four major toxins (α, β, ε and ι) (Cato et al., 1986; Petit et al., 1999). In the present study, only strains from toxinotypes A and C were found. The interpretation of this finding in rabbits is difficult since these toxinotypes are considered primary pathogens in necrotic enteritis only of poultry. The apparent correlation between the presence of ERE gross lesions and detection of *Cl. perfringens* alpha toxin in the digestive contents of affected rabbits should not be misread. Indeed in experimentally induced ERE, high *Cl. perfringens* counts are only observed in 1/10 animals and in 1/5 control animals (D. Marlier and H. Vindevogel, unpublished data). Thus, both the high *E. coli* and *Cl. perfringens* faecal counts and putative links between the alpha toxin and gross lesions might well reflect the intestinal disorders only, without being the cause.

Viral digestive diseases of rabbits are still poorly studied. Some viruses (adenovirus, coronavirus, parvovirus and rotavirus) have been found in the digestive contents of rabbits that died of digestive disorders (Nieddu et al., 2000; Baker, 1998). By electron microscopy, the presence of viral particles has been found in 37.3% of samples from rabbits suffering from intestinal disorders with the most frequent viral species being rotavirus (41.9%), coronavirus (25.6%), parvovirus (21.1%) and enterovirus (10.3%) (Nieddu et al., 2000). Among these, only rotavirus could be a primary pathogen (Thouless et al., 1988). Rotaviruses are endemic in most rabbitries in which they only induce mild digestive disorders unless complicated by supervening bacterial infections (Thouless et al., 1996). Rotaviruses do not seem to be the cause of ERE since they were only detected in 33% of rabbits naturally infected by ERE. The hypothesis that another viral family may be involved cannot be rejected.

A slight infection with *Eimeria* spp. was observed in 40% of cases without coccidiosis gross lesions. Subclinical infections with coccidia of the genus *Eimeria* frequently occur in rabbits (Peeters et al., 1984); furthermore, there is a marked variation in the pathogenicity among the different species of intestinal coccidia (Varga, 1982). In the present study, no *Cryptosporidium* oocysts were observed. In Belgium, oocysts were detected in 2–11% of weaned diarrhoeic rabbits. Inocula-

tion of weaned rabbits causes no mortality and only very discrete diarrhoea was induced (Peeters et al., 1986). In any case, the direct intervention of *Cryptosproridium* spp. in the aetiology of ERE seems unreliable since the aetiological agent is able to withstand at least two unprotected freeze-thawing cycles without lost of infectivity (D. Marlier and H. Vindevogel, unpublished data), a process that would kill *Cryptosporidium* oocysts (Tzipori, 1983).

No specific histopathological examinations of field cases were included in this study. Indeed, from the start it was evident that in such cases the potentially specific lesions induced by the putative ERE aetiological agents were hidden by unspecific lesions due to secondary bacterial infections developing during the course of ERE (D. Marlier and H. Vindevogel, unpublished data). Tissues of the gut are very susceptible to post mortem desquamation of the epithelium, making a correct diagnosis very tricky. Wyers (2003) studied the microscopic lesions in SPF inoculated rabbits and confirmed the lack of inflammatory lesions but failed to identify any specific lesions of ERE. This was also the case in the histopathological sections from the ten inoculated-rabbits we analysed. Since only photonic microscopy was used, we performed a study dedicated to the comparison of the microscopical lesions in sections from the different parts of the intestinal tract by light microscopy (specific staining for intracellular organisms), scanning electron microscopy and transmission electron microscopy (D. Marlier, unpublished data). The presence of bacteria either adhering to the epithelial surface or within epithelial cells was observed in some animals by transmission electron microscopy (rods with an undulating outer membrane about 1500 nm in long and 700 nm in width) and by light microscopy following Warthin–Starry staining. These bacteria are supposed to be uncultivable. An aetiological role, if any, for these bacteria in ERE remains to be demonstrated.

The inoculation experiments show that the ERE can be experimentally reproduced using digestive contents from affected rabbits. The Belgium B1 and B2 inocula seemed less virulent than the French TEC3 inoculum. Indeed, the mortality rates, the clinical signs, and the gross lesions were more severe with TEC3. A possible explanation could be a greater number of freezing-thawing cycles undergone by B1 and B2 leading to a loss of virulence. The mortality rate observed in SPF rabbits (15–33%) was lower than in naturally infected animals (30–80%) (Licois, 1998), which suggests that secondary infection plays a key role in the field. The disease was not reproduced with the different pools of bacterial strains and the measured DWG remained high in the different groups. This could mean either that none of the bacterial strains was an aetiological agent or that the isolated strains had lost their virulence.

5. Conclusion

No final aetiology of ERE has been identified. However, the successful reproduction of the disease using intestinal contents strongly suggests that the aetiological agent(s) is or are present in the different inocula used. In the absence of a well-identified aetiology, the confirmation of ERE in field cases still remains a problem and must be based on the observations of: (1) high mortality rates in 6–14-week-old rabbits with (2) huge abdominal distension reflecting (3) the distension of stomach and small intestine, (4) without evidence of inflammatory lesions and (5) with no concomitant infection with specific rabbit pathogens.

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