Studies on the evolution, pathology, and immunity of commercial fattening rabbits affected with epizootic outbreaks of diarrhoeas in Mexico: A case report

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Abstract

Epizootic outbreaks of diarrhoeas have emerged and disseminated in different rabbit farms in Mexico causing great economical losses, during the past years. Seven, 5-weeks-old New Zealand White (NZW) rabbits chosen at random from 35 ill animals that were remitted for postmortem, histopathology, and ultrastructural examinations were studied. Bacteriological and parasitological studies were carried out in three additional ill rabbits of same age. In a field trail 45, 5-weeks-old apparently healthy NZW rabbits were observed daily for sanitary status for a 5-week period. Some of the rabbits did not response to the preventive drug treatment and were therefore, used to study the development of the disease. Clinical signs, gross lesions, and mortality throughout the fattening period were recorded. Eight, 8-weeks-old NZW rabbits who survived an outbreak were assessed for gamma-globulins in serum of the total protein fraction during a 3-week period. Gamma-globulins were also measured in eight free-disease healthy rabbits of same breed and age. Lesions of the small intestine consisted of mucoid enteropathy, lymphocytic plasmocytic enteritis with atrophy and fusion of villi, and hyperplasia of globet cells. Serosal edema was present. Ultrastructural examinations of jejunum and ileum from 3/7 diseased rabbits, revealed enterocytes in apoptosis, mixed with degenerative and/or necrotic changes together with infiltration of lymphocytes, macrophages, neutrophils, and loss of microvillus. There were electron dense structures suggestive of virus particles inside the nuclei and cytoplasm of some enterocytes. There was lymphoid spleen atrophy and proliferation of reticuloendothelial cells in 7/7 rabbits. Interstitial pneumonia in 4/7 rabbits was found. *Encephalitozoon cuniculi* was detected in the brain of 1/7 rabbits. *Escherichia coli* were detected in 3/3 cases and *Eimeria* spp. in 2/3 cases. Mortality rate in the field study was 51.1% and the spread of the disease occur in 9/9 cages. The proportion of gamma-globulins in rabbits who survive an outbreak was much lower ($P = 0.0001$) than free-disease healthy rabbits (8.1 ± 1.0 and 14.0 ± 1.0, respectively). The disease was multifactorial and consisted of sub-acute mucoid enteropathy probably induced by viral infection and aggravated by the proliferation of opportunistic pathogens common to rabbits. This may explain the severe degenerative and necrotic changes observed in the small intestine of diarrhoeic rabbits.
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Keywords: Mucoid enteropathy; *Escherichia coli*; Virus-like particles; Hypogammaglobulinemia; Rabbits

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1. Introduction

During the late 2001 and early Spring of 2002, a sharp increase in the incidence of epizootic outbreaks of diarrho-

eas characterized by a high morbidity and mortality occurred in different commercial rabbit farms in Mexico. The disease disseminated very rapidly in different counties causing considerable economical loss. Affected rabbits aged 5–7 weeks manifested apathy, depression, decreased feed intake, abdominal bloating, limited watery diarrhoea sometimes with mucus, dehydration, and death. The disease was characterized as sub-acute mucoid enteropathy with unusual high rates of mortality. Death rates during the fattening period varied from 16.7% to 35.2% depending on the time of the year, type of commercial diet, preventive antibiotherapy strategy, animal density, and hygienic conditions (Rodríguez-De Lara, 2003). The disease was highly transmissible and presumptive immunosupprression was suggested. Some other field studies on the characterization and lesions observed in rabbits showing severe cases of diarrhoeas in Mexico have been published (Salcedo-Baca et al., 2004; Ventura et al., 2004). In a recent report study, Beltz et al. (2005) found, in different segments of the small intestine, a sub-acute lymphocytic–plasmocytic enteritis with atrophy and fusion of villi and globet cells hyperplasia.

Diarrhoeas in rabbits represent an important factor influencing profitable rabbit production. The aetiology of mucoid enteritis is unknown and has been referred as mucoid enteritis complex because causes are often multiple (Whitney, 1976). However, in true cases of mucoid enteritis, visible inflammation of the intestine was not observed, and instead was designated mucoid enteropathy (ME) complex (Flatt et al., 1974). Symptoms of ME consist of depression, anorexia, abdominal bloating, diarrhoea, mucus in faeces, hypothermia, and dehydration. At necropsy the stomach and small intestines are dilated, thickened, and filled with translucent mucoid fluid. The caecum appears impacted and the sacculated portion of the colon presents clear gelatinous mucus. Hyperplasia of globet cells throughout the small intestine is a feature (Van Kruiningen and Williams, 1972). Lesions in the caecum were minimal to absent (Whitney, 1976). Mucoid enteropathy has been related to gut dysfunction or due to direct pathogenic agents. A wrong feed formulation, season, excessive antibiotics administration, stressors, loss of passive immunity, cold, and early weaning induce changes in the gastrointestinal motility and in pH conditions resulting in abnormal caecal fermentation and proliferation of pathogens agents which co-existing at the same time caused diarrhoeas (Laplace, 1978; Lelkes and Chang, 1987).

In commercial rabbits, the involvement of highly pathogenic agents acting synergistically with moderate endemic pathogens results in multifactorial enteropathy difficult to diagnose and treat (Boucher and Nouaille, 1996). Bacterial enteritis can be caused by infection with enteropathogenic Escherichia coli (EPEC), Clostridium piliformis (causative of Tyzzer's disease), Clostridium spiriforme (causative of iota-toxin enterotoxemia), Salmonella spp., Kleibsella, Pseudomonas, and Campylobacter-like species among others (Peeters, 1987; Licois, 1989; Boucher and Nouaille, 1996; Marlier et al., 2003a). Parasitic disorders of the gastrointes-tinal tract of the rabbit involve infection with Eimeria spp., Cryptosporidium spp., Passalurus ambiguus, Passalurus nonanulatus, Obeliscoides cuniculi, and a variety of cestodes and trematodes (Brooks, 1983; Marlier et al., 2003a). However, intestinal coccidiosis are the most frequent parasites isolated when diarrhea occurs and have been closely associated with ME complex (Peeters, 1987; Licois, 1989; Marlier et al., 2003a). The severity of coccidiosis has been related to the species involved and to the level of infection. According to Peeters (1988), intestinal species include those with high pathogenicity (E. intestinalis, E. piriformis, and E. flavescencs), moderate pathogenicity (E. magna, E. media) and low pathogenicity (E. perforans, E. irresidua, and E. coecicola). While different studies related to the participation of virus in episodes of mucoid enteritis-complex in rabbits also exist, rotavirus are the most commonly virus detected in cases of diarrhoeas in weanling rabbits (Schoeb et al., 1986; Peeters, 1987; Licois, 1989; Marlier et al., 2003a). Experimental infection with adenovirus, parvovirus, and coronavirus could cause very mild symptoms and moderate enteritis in the small intestines (Bodon and Prohaszka, 1980; Matsunaga and Chino, 1981; Descoteaux et al., 1985). The co-existence of different enteric pathogen agents causes severe destruction of the mucosal integrity resulting in anorexia, diarrhoea, dehydration, polidypsia, and death (Wilber, 1999; Nieddu et al., 2000).

Little is known on the nature, spontaneous evolution, and pathology of the mucoid enteropathy syndrome observed in rabbit farms in Mexico and there is a great concern among producers because of the disease's persistence and continuing dissemination, as well as for great similarity with the epizootic rabbit enteropathy (ERE) described in commercial units in Europe (Licois, 1998; Vandekerchove et al., 2000; Licois et al., 2000; Licois, 2002; Marlier et al., 2003b; Licois et al., 2005). The ERE has been described as a digestive syndrome associated with high mortality and rapid spread affecting mainly young fattening rabbits. It is characterized by depression, decreased feed intake, abdominal distension, limited diarrhoea, and sometimes discharge of mucus (Licois, 1998; Licois et al., 2000). Gross lesions include the stomach and intestines which are distended due to the presence of abundant gas and fluid. At necropsy, rabbits with ERE are absent of any inflammatory or congestive lesions but show signs associated with caecal paresis and the presence of variable amounts of gelatinous mucus particularly in the colon and sometimes in the small intestine. Epithelial lesions of the small intestine are particularly marked towards the ileum and characterized by a proliferation of glands, prominent generalized atrophy of villi, and features of epithelial degeneration and necrosis which are most often slight and disseminated. Epithelial lesions in the large intestine are degenerative and slight. A congestion and villus atrophy of duodenum, jejunum, and ileum with a diffuse mucosal lymphoid infiltrate in rabbits affected by a naturally occurring ERE have been reported (Vandekerchove et al., 2000).

Several authors have claimed to isolate EPEC, *Clostridium* spp., *Eimeria* spp., and rotavirus in episodes of ERE in different rabbit farms in Europe (Rossi et al., 1999; Licois et al., 2000; Coudert et al., 2000; Dewree et al., 2003; Marlier et al., 2003b). Nieddu et al. (2000) in Italy detected viral particles by negative staining electron microscopy from rabbits with enterotyphylitis, which varied from catarrhal to catarrhal-haemorrhagic and in some cases to necrotic-haemorrhagic lesions. The presence of at least one type of viral particle was detected in 37.3% of faecal material of deceased rabbits presenting digestive pathology. Viral particles similar to rotavirus (41.9%), coronavirus (25.6%), parvovirus (21.1%), and enterovirus-like virus (10.3%) were found. In most cases, multiple infections with rotavirus-like particle, coronavirus, and parvovirus were mainly involved. Legall et al. (1998) supported the hypothesis of viral infection as the main cause of ERE. In a recent study, Licois et al. (2005) demonstrated that the intestinal content from ERE-rabbits was infectious as early as the second day, but the aetiological agent was not identified.

Studies were undertaken in an affected rabbit farm in Mexico manifesting severe cases of diarrhoeas in an attempt to generate information about the aetiology and to relate the isolated pathogen agents with clinical signs, gross lesions, histopathology, electron microscopy, bacteriology, parasitology, and mortality. Of particular interest was to determine the proportion that serum gamma-globulin comprised of the total protein fraction during the late fattening period of rabbits, who survived the acute phase of the disease vs. that found in disease-free healthy rabbits. This may help to evaluate presumptive immunosuppression.

2. Material and methods

2.1. Location

The animals used in the present studies came from the Rabbit Research Center (Centro de Investigación Científica del Estado de Mexico A.C.) located in San Miguel Coatlinchan, Texcoco in the Valley of Mexico at 19′27″ and 98′53″ at 2220 m above sea level. The mean annual temperature is 15 °C with 645 mm of rainfall per year.

2.2. Centre housing facilities

The rabbit centre facilities consisted of a maternity unit and a fattening area with the capacity to keep 180 breeding does and 1176 fatteners, respectively. The animals used in the present investigations were kept in a fattening unit 25×10 m with natural ventilation and thermal insulation. All rabbits were maintained in $90 \times 60 \times 40$ cm individual wire cages in a flat-deck system, provided with automatic watering and two J-feeders (Jaulas Lopez Hermanos, Mexico, DF) per cage with 1500 g feed capacity each. Young animals were maintained under natural lighting.

2.3. Case history

During the late winter of 2002, the entrance of practitioners for 2 days was coincident with the spread of an emergent disease in the centre. The practitioners had previously been working in some rabbit units affected with severe cases of diarrhoeas, without notifying the centre. The practitioners wore the same clothes and boots used in the other farms. One week later, an outbreak of severe diarrhoeas in the fattening unit killed 173 young animals within a 2-week period. Breeding does were also affected. Since the entry of the disease and before depopulating the centre in August 2003, the outbreaks of diarrhoeas persisted and studies on the spontaneous evolution, pathology, and immune status of rabbits were performed.

2.4. Animals and laboratory examinations

Seven clinically ill New Zealand White rabbits of 37 days of age were sent to the Department of Pathology of the Faculty of Veterinary Medicine at the National University of Mexico for postmortem, pathology, and ultrastructural examinations (lab 1). Typical histories described for all remitted rabbits were severe emaciation, bloated abdomen, limited watery diarrhoea, and, in some, mucus diarrhoea. Rabbits were chosen at random from 35 ill animals during the acute phase of an outbreak of severe diarrhoeas. The rabbit population in the fattening unit at this time was approximately 735 animals. Three additional rabbits from a subsequent outbreak were submitted to the Laboratory of Microbiology of the Department of Animal Husbandry of the Autonomous University of Chapingo (lab 2) for microbiological and parasitological examinations. The differential diagnoses were colibacilosis, clostridiosis, salmonellosis, campylobacterosis, viral agents, intestinal and hepatic coccidiosis, and helminth parasites.

2.5. Histopathology

Seven rabbits were sacrificed through cervical dislocation. Sections of duodenum, jejunum, ileum, caecum, colon, lungs, liver, spleen, kidneys, adrenal glands, myocardium, cerebellum, and cerebrum from necropsy were fixed within 20 min after sacrifice in 10% buffered formalin for 48 h. Samples were embedded in paraffin and sections at 5 μm thickness were cut and stained with haematoxylin–eosin. Periodic acid–Schiff stain, Grocott methanamine silver, and Ziehl–Neelsen acid-fast stains were done to detect mycosis or acid–alcohol resistant bacteria in lung tissues.

2.6. Ultrastructure

Samples of small intestine from 3/7 rabbits were selected for ultrastructural examination to locate virus particles and other changes in enterocytes. Samples were cut into 2–3 mm³ and then washed with sodium cacodylate buffer (pH 7.4, 0.1 M). After this procedure, samples were post-fixed in

osmium tretoxide (OsO_4, 1%). Samples were dehydrated in an ascendant serial ethanol and embedded in epoxy resin (Epon 812). Finally, tissues were cut 70–80 nm thick and stained with uranyl acetate and lead citrate. Thin sections were examined under a Zeiss EM 900 (50 kV) transmission electron microscope.

2.7. Bacteriology

Stool samples from three ill rabbits during the acute phase of the disease were collected and remitted to lab 2 for microbiological media cultures. Ten grams of each sample was placed in a sterile stomacher bag containing 90 mL of peptone water. *Escherichia coli* were found on modified eosin methylene–blue agar (Oxoid CM69), Mac Conkey agar (Oxoid CM7), and blood agar base (Oxoid CM55) aerobically and incubated at 37 °C for 24–48 h. To isolate *Campylobacter* spp. samples were culture in *Campylobacter* selective supplements (Skirrow) (Oxoid, SR69). Plates were incubated at 42 °C during 18–24 h. For *Clostridium* spp. samples were cultured in reinforced clostridia agar (Oxoid CM151) and incubated at 37 °C for 24 h in an anaerobic environment. For detection of *Salmonella* spp. stool samples were plated in brilliant green agar (Oxoid CM263) and incubated during 24 h at 35 °C.

2.8. Feed microbiology

Samples of food taken from different cages occupied by ill rabbits were collected and remitted to lab 2. To assess the microbiological quality, the following analyses were performed: mesophilic bacteria counts and counts of coliform. Counts for mesophylic aerobics bacteria were verified by plating serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}) on plate count agar (Oxoid roll tube) and counting the colony forming units after incubation. Samples were analyzed for total coliforms according to the most probable number (MPN). Number of coliforms per gram was estimated through the combination of positive and negative tubes. Briefly, 10 g of food samples were homogenized and then serially diluted 10-fold. From all five consecutive dilutions (from 10^0 to 10^{-4} or 10^{-1} to 10^{-5}), 1 mL was poured to each three violet red bile (VRB) broth media (Oxoid CM107) and incubated at 37 °C for 24–48 h. Gas positive tubes were identified. Then positive tubes of three consecutive dilutions were transferred into brilliant green bile (2%) broth (Oxoid CM31) and incubated at 37 °C for 24 h for the confirmation of presumptive coliform counts obtain from VRB broth.

2.9. Parasitology

The granulomatous encephalitis found in the brain of one rabbit in lab 1 indicated the possible presence of *E. cuniculi*. Consequently, additional brain tissue of the same rabbit was smeared with Giemsa's stain for microscopic appreciation. Faeces from three ill rabbits were analyzed by direct microscopy and flotation (Felk and Moody, 1993) and assessed according to the literature (Georgi and Georgi, 1990).

2.10. Field study

Forty-five apparently healthy New Zealand White weanling rabbits aged 35 days were provided with a preventive drug treatment for 5 days and observed daily for sanitary status for a 5-week period. Some of the rabbits did not respond to the treatment and were therefore, used to study the development of the disease. Clinical signs, gross lesions, and mortality throughout the fattening period were recorded. The present study was carried out the late winter and early spring of 2003. The animals were kept under optimal environmental conditions in the rabbit centre housing facilities and allocated in nine cages distributed in a batch. Five rabbits were kept in each cage. The cages and the automatic watering system were cleaned and disinfected with synthetic phenol solution (Ambrientrol, Norvatis). Immediately after weaning, the rabbits received 10 mg/kg of body weight of enrofloxacin (Centryl 5%, Parfam) given in the drinking water every 24-h during 5 days period. A commercial pellet diet was used, containing 88.7% dry matter, 16.5% crude protein, 3.5% fat, 23.9% acid detergent fibre, 42.4% neutral detergent fibre, 10.0% ashes, and 2200 kcal of digestible energy per kg (DM basis). Young rabbits were fed *ad libitum*. Necropsies of dead rabbits were performed to assess gross lesions.

2.11. Gamma-globulins in serum assays

Eight New Zealand White rabbits aged 8 weeks who survived an epizootic outbreak of diarrhoeas were isolated and gamma-globulin proportions of the total protein serum fraction were assessed every 7 days during a 3 weeks period. This study was performed at the beginning of August 2003. In a further complementary study carried out in August of 2005, eight healthy rabbits of the same breed and age provided from a farm never affected by diarrhoeas were kept in environmental conditions similar to affected rabbits. Thereafter, serum gamma-globulins levels were assessed. The healthy rabbits were used as control group. In the two trials, rabbits were kept under a natural ventilation system. All animals were allocated individually in cages $20 \times 46 \times 41$ cm cages provided with automatic watering system and English J-feeders. The animals did not receive any antibiotic or desparasitant and feeding was the same. Three millilitres blood samples from each rabbit were obtained by vein puncture in 1 day between 9 and 10 a.m. Blood collection was carried when rabbits aged 8, 9, and 10 weeks. No anticoagulant was used in blood collection. The serum was obtained by centrifugation of the blood sample under 1200 rpm during 15 min. Gamma-globulins were measured using cellulose-acetate electrophoresis described in the manual of the spectrodensitometer (Cellomatic Junior).

2.12. Statistical analysis

The proportion of gamma-globulins in serum was analyzed after repeated measures by proc MIXED (SAS, 2001) and was compared before and after arccosine transformation. Statistical results were similar, therefore untransformed data are presented. Means were compared by Tukey's test. The model included the fixed effect of health status (survivors and healthy rabbits), the age of does (8, 9, and 10 weeks), and their two way interaction. The model was: $Y_{ijk} = \mu + H_i + A_j + (H*A)_{ij} + e_{ijk}$ where: Y_{ijk} is any value of the response variable; μ is the overall mean; H_i is the fixed health status effect (i = survivors, healthy rabbits); A_j is the fixed effect of age of does (j = 8, 9, and 10 weeks of age); $(H*A)_{ij}$ is the interaction between health status of rabbits and age; e_{ijk} is the random effect $\sim NI(0, \sigma_e^2)$.

3. Results

3.1. Clinical findings

The main clinical manifestation of most rabbits was gastrointestinal problems with varying degrees of severity. Signs consisted of anorexia, lethargy, distension of the abdomen (6/7), caecal impaction (6/7), diarrhoea (6/7), rumbling noise (6/7), mucus excretion (1/7) and dehydration (6/7). One rabbit presented weakness.

3.2. Gross lesions

At necropsy, the rabbits had a regular flesh (6/7) to poor nutritional condition (1/7). Two rabbits presented the perennial region stained with greenish-brown semi-liquid stools. Gross post mortem examination in six rabbits revealed a thickening of the intestinal mucosa wall. There was abundant greenish-brown fluid content mixed with foamy mucus inside the small and large intestines. The content of the caecum in these rabbits was partly desiccated. Notable distension of the caecum was observed due to gas and fluid. The mesenteric vessels were notably visible as the result of congestion. Rabbit D presented a clear and copious gelatinous translucent mucus obstructing different segments of the colon. There were no congestive or inflammatory macroscopic lesions in the caecum. There were no significant gross changes in the other examined organs.

3.3. Histopathology

Histological findings were mainly prominent in the gastrointestinal tract and spleen. Almost all the rabbits (6/7) had lesions in duodenum, jejunum, and ileum with varying degrees of severity (Table 1). In the caecum there was far less damage in the epithelium. Epithelial lesions of the small intestine were mainly atrophy and fusion of villi, and slight to moderate diffuse infiltration of lymphocytes and plasma cells along the mucosa. Different intestinal lesions observed in some rabbits are depicted in Fig. 1.

Lesions in the duodenum were present in 7/7 rabbits. Most affected rabbits had moderate to severe inflammatory infiltrate in this part of the intestinal mucosa. In most cases, the lymphocytes and plasma cells were the predominant cell types involved. Three rabbits had diffuse atrophy and fusion of villi of varying severity. The lesions ranged from moderate to severe. All the seven rabbits had diffuse hyperplasia and hypertrophy in globet cells from mild, moderate or mild to moderate or moderate to severe.

Table 1
Histological lesions in the intestine and spleen in the examined rabbits

Rabbit/organ and lesions	A	B	C	D	E	F	G
Duodenum							
Lymphocytic and plasmocytic infiltrate	+ to ++	+ to ++	+ to ++	++ to +++	++	++	+ to ++
Atrophy and fusion of villi	++	–	–	–	++	++ to +++	–
Hyperplasia of goblet cells	++	++	–	+ to ++	++	++ to +++	+
Jejunum							
Lymphocytic and plasmocytic infiltrate	+ to ++	+	++	+++	++	++	+ to ++
Atrophy and fusion of villi	++ to +++	+++	++	++ to +++	++	–	–
Hyperplasia of goblet cells	++	++	++	++	+	++	+
Ileum							
Lymphocytic and plasmocytic infiltrate and hyperplasia of lymphoid tissue	++	++	++	++	++	++	+ to ++
Atrophy and fusion of villi	++	++	++	++	++	+	+
Hyperplasia of goblet cells	+ to ++	+	+	+	++	+	++
Spleen							
Lymphoid depletion	++	++	++	+++	++	++	++

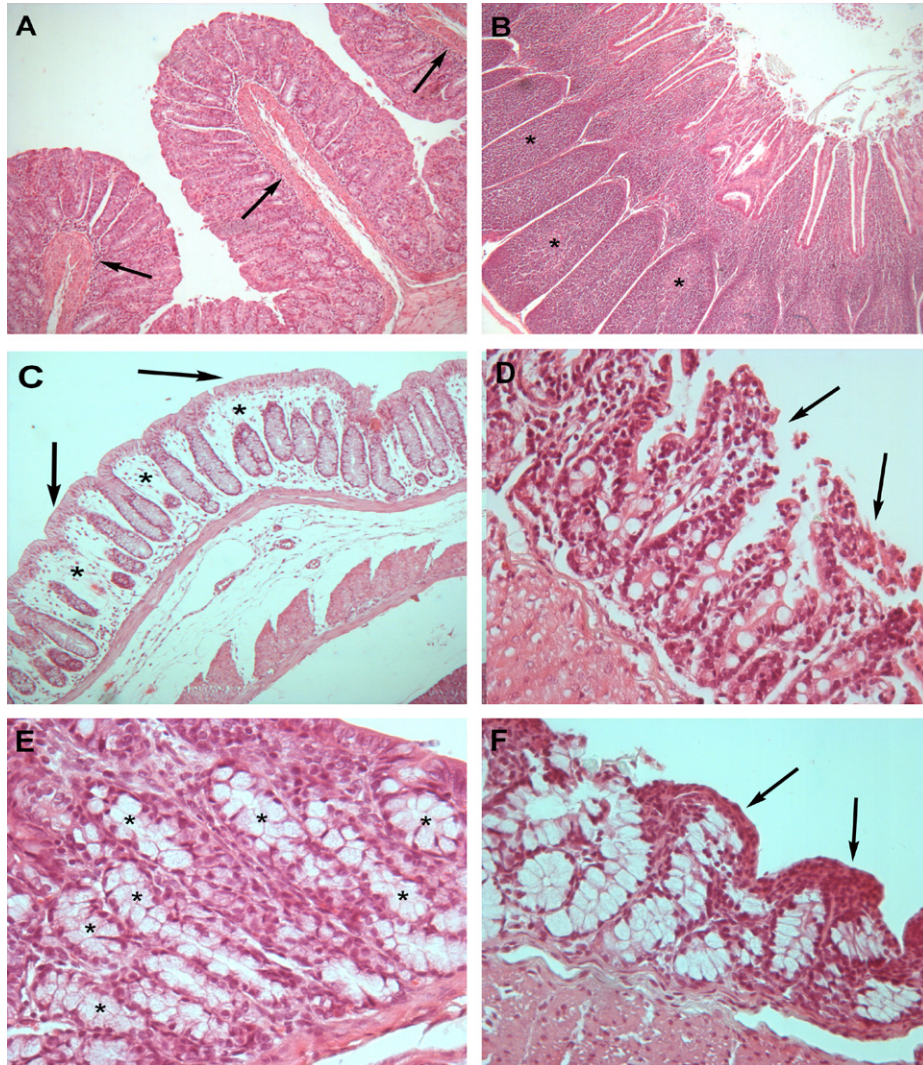(−) not present, (+) mild, (++) moderate, (+++) severe.

Fig. 1. Selected views of pathological lesions in the intestinal mucosa of some rabbits. (A) Mild diffuse infiltration of lymphocytes and plasma cells (arrows) along the intestinal mucosa of rabbit B (100×, HE). (B) Severe hyperplasia of lymphoid tissue (*) with marked infiltration of lymphocytes along the intestinal mucosa of rabbit G (40×, HE). (C) Diffuse moderate edema (*) in the intestinal mucosa of rabbit F with marked atrophy and fusion (arrows) of villi (100×, HE). (D) Severe atrophy and fusion of villi with sloughing of epithelial cells (arrows) and mild infiltration of lymphocytes in the intestinal mucosa of rabbit A (400×, HE). (E) Marked hyperplasia of globet cells (*) along the intestinal mucosa of rabbit E (400×, HE). (F) Squamous metaplasia in the apix of villi (arrows) in the intestinal mucosa of rabbit D with marked atrophy and fusion of villi and severe hyperplasia of globet cells (400×, HE).

Similarly, pathological changes in the jejunum included 7/7 rabbits. Most affected rabbits had diffuse infiltration of lymphocyte and plasma cells in the mucosa being mild, moderate, severe or mild to severe. In one case, a mild infiltrate of lymphocytes and plasma cells in the base and tip of the villi was present. Atrophy and fusion of villi occurred in five rabbits and ranged from moderate, severe or moderate to severe. In rabbit D, squamous metaplasia in the apix of villis in the jejunum mucosa with marked atrophy and fusion of villi and severe hyperplasia of globet cells were observed. Five rabbits had moderate, diffuse hyperplasia and hypertrophy of globet cells in this segment of the small intestine. In another two cases, lesions were mild and diffuse. Along the mucosa in the jejunum in rabbit E, there was a marked hyperplasia of globet cells, atrophy and fusion of villi and a moderate infiltration of lymphocyte.

The lesions observed in the wall of the mucosa of the ileum of 7/7 rabbits also consisted of infiltration of inflammatory cells. The lymphocytes and plasma cells were the predominant cells involved. In six rabbits, the inflammatory process was moderate and diffuse, whereas in other case the lesion was mild to moderate and diffuse. In one rabbit there was mild infiltration of lymphocytes and plasma cells of enterocytes at villar tips and mid location. In three of these cases, a moderate and diffuse inflammatory process towards the tip intestinal villi was observed. Atrophy and fusion of villi was revealed in 7/7. This lesion was moderate and diffuse in five rabbits and mild, diffuse in two cases. Four rabbits had mild and severe hyperplasia and hypertrophy of globet cells. In three cases lesions were diffuse and ranged from mild to moderate or moderate. Hyperplasia of lymphoid intestinal tissue was observed in most cases.

Depletion of the white pulp in moderate or severe quantities and proliferation of reticuloendothelial cells was a feature in the spleen of all rabbits. Microscopic lung lesions were detected in 4/7 rabbits. The alveolar septums of all of these rabbits were thickened due to moderate infiltration of inflammatory cells mainly lymphocytes and plasma cells. In two of these rabbits, the alveolar spaces were occupied by great amount of epithelial macrophages and some slight multinucleated or syncytial cells in alveolar spaces were present. A notably distension of alveolar cells and ruptured alveolar septa or emphysema in some areas was a feature in three rabbits. Lung lesions from three rabbits were characterized by discrete to moderate multifocal lymphoid hyperplasia in the peribronchiolar region. In the cerebellum, there were necrotic focis; glyosis and inflammatory infiltrate mainly lymphocytes and macrophages.

3.4. Diagnosis

All the rabbits had lesions in the small intestine (7/7). However, the mucosa and villi of the jejunum and ileum appeared to be the most affected. Based in the histology finding, there was diffuse lymphocytic and plasmocytic enteritis. These epithelial lesions ranged from mild, moderate or mild to moderate or moderate to severe. Atrophy and fusion of the small intestinal villi, diffuse was a feature in all cases. These lesions were diffuse and ranging from mild, moderate, severe or mild to severe. There was a diffuse hyperplasia of globet cells ranging from mild, moderate or mild to moderate or moderate to severe in most rabbits.

Moderate to severe, diffuse lymphoid spleen depletion and atrophy were prominent features in all rabbits. A marked proliferation of reticuloendothelial cells in the spleen was also observed in these rabbits.

Microscope lesions were present in the lungs of 4/7 rabbits. These lesions consisted of mild to moderate interstitial pneumonia. A moderate diffuse lymphoproliferative, sloughing of epithelial cells, lymphocytic, hystiocytic multifocal pneumonia was also observed in two affected rabbits. In two rabbits, syncytial cells in alveolar spaces were observed. In one rabbit, zonal and acute moderate intrapulmonary haemorrhage was revealed. One affected rabbit also showed severe zonal parenchyma haemorrhage.

Mild to moderate, diffuse brain congestion was a feature in 5/7 rabbits. In one case, multifocal, mild granulomatous encephalitis was observed. Severe acute, zonal haemorrhage was observed in another affected rabbit in the meningeal tissue.

3.5. Ultrastructure

Examinations of jejunum and ileum, revealed similar changes with the same degree of severity. At the mucosa, there was hyperplasia of globet cells with an increase in their production. In the intestinal lumen, material of granular-like aspect of moderate electron density mixed with cellular debris, bacteria, and yeast in close association with micro-villus were observed. Several multifocal enterocytes, with or without presence of bacteria, showed a partial loss of villi varying from mild to moderate. Abundant Gram-negative bacilli were observed in the villi. We found enterocytes with different phases of apoptosis mixed with degenerative and/or necrotic changes randomly distributed. These changes varied from mild to moderate multifocal and showed the presence of lymphocytes, macrophages, and neutrophils. Inside the nuclei of some enterocytes were numerous electron dense structures with almost spherical to hexagonal shapes of different sizes ranging from 121 to 260 nm of diameter. The pseudo nuclear like inclusion was surrounded by a true nuclear membrane. These virus-like particles were in cumulus of 4–32 structures. The presence of electron dense structures inside the nuclei of one enterocyte is shown in Fig. 2. In some enterocytes, there were numerous cytoplasmatic electron dense structures randomly distributed with similar characteristics to those found inside the nucleus. There were electron dense structures inside the nuclei of cells with round to oval shape located in the lamina propria. They consisted of spherical electron dense structures of 122 nm of diameter that were compatible with virus-like particles. These particles were identical to those found in the enterocytes of the small intestine.

3.6. Bacteriology

Escherichia coli was isolated in abundance in stool samples from two rabbits with traces in the other. However, pathogenic sub-type was not determined. All the rabbit stool samples investigated were negative for *Campylobacter* spp., *Salmonella* spp., and *Clostridium* spp. isolation.

3.7. Feed microbiology

The feed appeared normal in consistency, quality, colour, smell, and taste. Microbiological examination of the feed revealed mesophylic aerobic and coliform bacteria. The mesophylic aerobics accounted for 1.2 ± 10^6 colony forming units/g of the sample. The most probable number/g of sample was 93 faecal coliform bacteria.

3.8. Parasitology

In the brain tissue of one rabbit oval Gram-positive structures measuring 1.5–2 μm was observed within a parasitophorous vacuole. The parasite was identified as *E. cuniculi*. Parasitological studies were negative for coccidian and other potential parasites in one rabbit. The other two rabbits revealed moderate intestinal coccidian oocystes and no helminths. There was no presence of coccidian oocystes or helminths in liver in the examined rabbits.

3.9. Field study findings

Clinical signs and lesions evolved acutely. The disease affected young fattening rabbits between 7 and 9 weeks
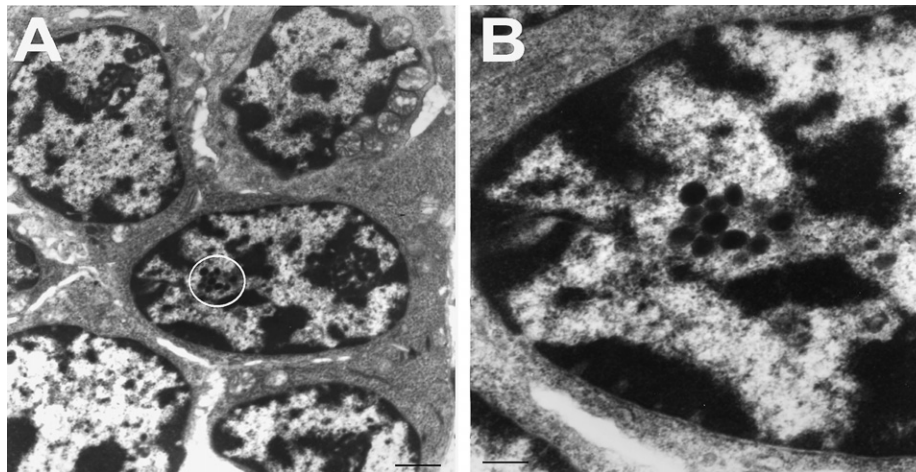
Fig. 2. Selected views of virus-like particles inside the nuclei of an enterocyte. (A) Presence of electron dense cumulus inside the nuclei (circle) of an enterocyte (7000×). (B) Detail of the previous photograph, in which round to oval structures of different sizes are disposed in cumulus suggestive of virus particles (20,000×).

of age. The mean percentage of rabbits dying during the fattening period was 51.1%. From 22 death rabbits, 95.5% manifested diarrhoeas while 4.5% showed poor body condition but no apparent clinical signs. The clinical signs and gross lesions of affected rabbits were similar to those observed in the animals remitted for laboratory examinations. Specific symptoms consisted of anorexia (21/22), depression (22/22), distension of the abdomen (21/22), rambling noise (21/22), diarrhoea of low intensity (21/22), presence of mucus in faeces (3/22), and dehydration (21/22). One rabbit presented non-specific signs but weakness. Symptoms evolved acutely and death occurred in 1–5 days after the onset of clinical signs.

There were not rabbit losses during the time of enrofloxacine treatment, but deaths occurred rapidly after its withdrawal. Depending on animal age, the mortality was 0%, 18.2%, 54.5%, 27.3%, and 0% for 6, 7, 8, 9, and 10 weeks old, respectively. One hundred percent of cages (9 of 9) were affected. The disease spread rapidly from one rabbit to others within the same cage and between neighbour's cages. A record of rabbits that died during the fattening period within and between cages along the fattening period since the onset of mortality is shown in Table 2. The onset of mortality was at day 10 after the beginning of the study. The period between the appearance of one dead rabbit and others within and between cages varied from 1 to 5 days with an overall mean of 2.7 days.

3.10. Gross lesions in field study

Rabbits were in good (1/22) to fair (21/22) postmortem condition. Twenty-one rabbits presented a pronounced abdominal swelling due to dilatation of all segments of the gastrointestinal tract. These rabbits presented moderate to abundant greenish-brown fluid content mixed with foamy mucus in the small and gross intestine. Clear and copious gelatinous translucent mucus obstructing different segments of the colon was prominent in three rabbits. The wall of the small intestine in 20/22 rabbits was thickening. The caecum of all rabbits was desiccated and notably distended due to the presence of gas and fluid. The mesenteric vessels were notably visible. In 6/22 rabbits, the different sections of the small and large intestine presented haemorrhages and ulcers in the caecum. In 2/22 rabbits, a copious discharge of mucus with blood were observed. No other gross significant changes were observed in other examined organs.

Table 2
Evolution of mortality of rabbits between and among cages from the onset to the end of rabbit losses

Cage	Days after onset of mortality																Total
	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
1								2	2		1						100
2							1							1			40
3					1											1	40
4	1																20
5				1													20
6				1		1								1	1		80
7										1		1	1				60
8										1							20
9										1	1		1				60

3.11. Gamma-globulins in serum

All 16 rabbits completed the two trials. Overall, mean proportion of gamma-globulins of the total serum protein fraction was 11.1. There was a significant ($P < 0.0001$) effect on health status of rabbits on this trait. The proportions of gamma-globulins in serum of rabbits who survived to an outbreak vs. healthy rabbits were 8.1 ± 1.0 and 14 ± 1.0, respectively. The proportion of gamma-globulins increased as the age of the rabbits increased ($P = 0.0005$). Mean proportion values for weeks 8, 9, and 10 weeks were 6.9 ± 1.2, 12.8 ± 1.2, and 13.4 ± 1.2, respectively. Differences were mainly due to age. The mean percentage of gamma-globulins in rabbits which survived the outbreak of diarrhoea for the 3 weeks period were 4.7 ± 1.7, 9.6 ± 1.7, and 10.1 ± 1.7, whereas mean values for disease free healthy rabbits were 9.1 ± 1.7, 16.1 ± 1.7, and 17.0 ± 1.7, respectively. There was no effect of interaction between health status and age of rabbits ($P > 0.05$).

4. Discussion

The clinical signs, gross, and microscopic lesions in gastrointestinal tract found in the present case, are similar to those reported in rabbits affected with sub-acute mucoid enteropathy in different rabbitries in Mexico (Salcedo-Baca et al., 2004; Ventura et al., 2004; Beltz et al., 2005) and resemble the old description of mucoid enteropathy complex described by several authors (Flatt et al., 1974; Van Kruiningen and Williams, 1972; Whitney, 1976). However, until the present study, the aetiological factors causative of the disease have not been defined. One of the most relevant differences between the old description of ME and the mucoid enteropathy reported in the present case is that the former acts as sporadic disease whereas, in the later, epizootic outbreaks with persistent and recurrent characteristics were manifested. The pathological changes are compatible with an enteropathy, but are not pathognomonic. Intestinal lesions were variable and appear to be non-specific but, in addition with the inflammatory infiltrate in the small intestine, suggest a viral infection; probably complicated with the presence of other enteric pathogens such as bacteria agents in rabbits. The fiber content of the pelleted diet was adequate. Rodríguez-De Lara (2003) observed that diet was not the main factor causing diarrhoeas in Mexico but acted as a predisposing factor when fiber content was inadequate. A low-fiber diet results in caecal-colonic hypomotility leading to constipation and diarrhoea (Laplace, 1978). Constipation is the result of disruption of the microflora of the rabbit gastrointestinal tract due to abnormal fermentations and proliferation of pathogen agents (Lelkes and Chang, 1987). The accumulation of mucus in the colon has been explained in terms of impaction, as the result of obstruction of transit due to intestinal stasis (Sinkovics, 1976) and this lesion has been experimentally induced by caecal ligation (Hotchkiss and Merrit, 1996). The direct action of pathogen agents constitutes

other cause of hypersecretion of mucus in the colon of rabbits (Licois, 2002).

The presence of high numbers of aerobic mesophilic bacteria and coliform in food from cages occupied by diarrhoeic rabbits indicates that unacceptable level of contamination occurred probably due to the co-existence of several rabbits in the same cage. Within the coliforms, E. coli is of great interest since their presence indicates faecal contamination with the possibility of accompanying other enteric pathogen agents. On the bacterial culturing, E. coli was isolated in abundant numbers in the faeces of two rabbits and traces in the other but the role of this bacteria is not clear especially when considering that E. coli are normal inhabitants of the gut and tests to determine pathogenic sub-types were not performed. Further steps will be taken to isolate and identified the biotype involved in rabbits with diarrhoea in Mexico to determine if sub-type involved is pathogenic.

Perhaps the long-time persistence of the disease in the centre, associated with reduced immunity and influenced by multiple predisposing factors, resulted in the establishment and proliferation of different opportunistic enteric pathogen agents leading to much higher levels of mortality than those reported in the same unit by Rodríguez-De Lara (2003). Salcedo-Baca et al. (2004) reported the presence of E. coli, Clostridium spp., Salmonella spp., Pasteurella spp., Glutaratus spp., Saccharomyces spp., and Eimeria spp. in a rabbit unit in Mexico affected with persistent severe cases of diarrhoeas for many years. The heterogeneity of pathogen agents found in different cases of epizootic outbreak of diarrhoeas in different rabbit farms in Mexico might be explained in terms of differences on the type of commercial diet used, animal density, husbandry, and hygienic conditions.

In our case, a moderate amount of coccidian was reported in two rabbits. Although tests to differentiate species were not carried out, our findings suggest that moderate to highly pathogenic species affecting the gastrointestinal tract might have a role in this finding, probably acting as complicating factor in the aetiology of diarrhoeas. However, this needs to be confirmed. The isolation of coccidias in cases of mucoid enteropathy have shown to be high (Peeters, 1987; Licois, 1989; Marlier et al., 2003a). Intestinal coccidiosis causes villous atrophy (Peeters et al., 1984) and reduces intestinal peristalsis (Fioramonti et al., 1981). According to Licois (2002), coccidiosis is responsible for digestive lesions similar to sub-acute atrophyant, erosive, and regenerative enteritis. Coccidian acting synergistically with other agents such as EPEC, Clostridium spp., and rotavirus cause diarrhoeas, high mortality, growth depression, and unfavourable feed conversion (Peeters, 1987; Nieddu et al., 2000).

The lymphocytic–plasmocytic enteritis with atrophy and fusion of the intestinal microvillus together with the degenerative and necrosis ultrastructural lesions suggest that mucoid enteropathy syndrome has complex multifactorial aetiologies including enteric viral infections. Rotaviruses

were detected in stool samples of rabbits showing epizootic outbreaks of diarrhoeas in Mexico (Salcedo-Baca et al., 2004; Beltz et al., 2005). In the present study, we were unable to detect rotaviruses. Nevertheless, a negative result does not exclude the possibility of rotavirus infection, especially if considering that rotaviruses are regarded as the most frequent detected virus in rabbits with diarrhoeas (Schoeb et al., 1986; Peeters, 1987; Licois, 1989; Nieddu et al., 2000; Marlier et al., 2003a) and that atrophy and fusion of villous in the present case correspond well with typical lesions caused by this virus (Thouless et al., 1988). Rotavirus, when introduced into colonies not previously exposed and with low immunity, results in a rapid onset and high mortality (Schoeb et al., 1986). However, rotaviruses as the main cause of the epizootic outbreaks of diarrhoeas in Mexico is questionable, particularly if considering that rotavirus are considered endemic and mildly pathogenic in commercial rabbitries (Peeters et al., 1984; Thouless et al., 1988). Despite this, rotaviruses could cause high death ratios when other viral infections and secondary pathogens like EPEC are involved (Thouless et al., 1996).

Particularly intriguing was the presence of numerous free virus-like particles inside the nuclei and cytoplasm of enterocytes of jejunum and ileum from 3/7 rabbits (42.8%). There are several reports that have pointed out the participation of virus in outbreaks in mucoid enteritis and rabbit enteropathies (Nieddu et al., 2000). Ultrastructurally in our case, the most striking changes were the hyperplasia of globet cells, random apoptosis and intranuclear and intracytoplasmic virus-like particles. Nevertheless, the morphology, size, and features of them were different from viruses described in the literature, associated with enteropathies in rabbits (Cheville, 1994; Murphy et al., 1999). The arrangement of the intranuclear components (heterochromatin, euchromatin, and nucleoli), as well as the integrity of the different organelles in the cytoplasm, suggest that there was absence or mild viral replication because otherwise there would have been a strong effect in nuclei and cytoplasm (Cheville, 1994; Murphy et al., 1999). Therefore, in our cases, it is strongly suggestive that the virus-like particles were in a latent stage or that they had finished their replication, when samples were taken.

Nieddu et al. (2000) carried out an electron microscopy study in rabbits with enteropathy, between the years 1982 and 1999. They found different virus particles in 37% of the cases. They were rotavirus-like, coronavirus, parvovirus, and enterovirus. They also described similar random particles such as adenovirus, calicivirus, and reovirus. These authors pointed out that more than one virus could be associated with mucous, haemorrhagic, and necrotic enteritis. On the other hand, different authors have associated rotavirus with the rabbit enteropathy, and it is considered an agent of moderate pathogenicity (Bryden et al., 1976; Peeters et al., 1984; Schoeb et al., 1986). Nieddu et al. (2000) pointed out that high mortality and economi-

cal losses can be important when other agents such as parasites and bacteria are associated with rotavirus.

Encephalitozoon cuniculi was diagnosed in the brain of one rabbit showing granulomatous encephalitis. This type of lesion is typically caused by this opportunistic microsporidum parasite (Pattison et al., 1971). The encephalitozoonosis has been also reported in others rabbit farms with severe cases of diarrhoea in Mexico but infesting the brain and kidney (Salcedo-Baca et al., 2004; Ventura et al., 2004). The presence of this parasite is rear but has been associated with stages of immunosuppression (Gannon, 1980) which appear to exist in the present case. Further study is needed to clarify many questions about this organism and the lesions with which it is associated.

A significant reduction in the proportion of serum gamma-globulins was observed in rabbits who survive to an outbreak of diarrhoeas (8.1%) when compared with free-disease healthy control rabbits (14%). The proportion of gamma-globulins assessed in surviving rabbits was shown to be below the 10% to 12% range values reported by Vaissaire et al. (1978) in 1–24 months old healthy rabbits. Results indicate persistently low gamma-globulins values for affected rabbits of 8, 9, and 10 weeks of age as compared with the healthy disease free rabbits. Mean values in the two groups increased as the age of the rabbit increased. The low levels of gamma-globulins in rabbits who survive an outbreak of diarrhoeas suggest defective host defences that are probably the result of a chronic disease, but the mechanism involved is unknown. For the high levels of mortality observed during the acute phase of the disease it is possible that low levels of gamma-globulins may also have occurred during this period however, this was not determined. The depletion of lymphocytes observed in the spleen of all rabbits during the acute phase of the disease in the histopathology study may be related with hypogammaglobulinemia that is probably due to a reduction in the number of T and B cells involved in the cellular and humoral immune response. However, a final conclusion cannot be drawn because functional analysis of the cells involved was not performed. Whether hypogammaglobulinemia was due to impaired B-cell antibody functions induced by virus is unknown, but intriguing since transient or more long-lasting generalized immune suppression has been documented with numerous viruses (Borrow et al., 1995). The 100% survival of rabbits manifesting low levels of gamma-globulins may be the result of low animal density encountered in the herd when the experiments were performed. This probably was accompanied by a substantial reduction in microbial contamination in the surrounding environment resulting in less risk of infection by other secondary opportunistic pathogen agents. Experimental evidence showed that when five rabbits who survived to an outbreak were allocated in the same cage under high animal density herd conditions, many of these rabbits died later during the late fattening period mainly due to digestive or respiratory disorders (Rodríguez-De Lara, 2003).

By its nature, the disease is strongly thought to be of infectious origin and highly contagious as supported by the 100% of cages affected and by the rapid spread pattern from one rabbit to another among and between cages. Apparently, horizontal transmission likely occurs through air-born particles, contact with contaminated surfaces, rabbits, excreta, urine, and probably through consumption of contaminated food. A temporary stop on the breeding programme in the maternity unit, depopulation of the fattening unit for 3 months and deep cleaning and disinfection was not a solution for eradicating the disease (Rodríguez-De Lara, 2003). As soon as the fattening unit was occupied, the disease recurred, suggesting that breeding stock may act as a asymptomatic carrier of hypothetical virus. Vertical transmission through the placenta and doe's milk may probably also occur, but this requires further investigation.

The general characteristics of the course and presentation of the outbreaks coincide with the description of the European outbreaks of epizootic rabbit enteropathy (Licois, 1998; Vandekerchove et al., 2000; Licois et al., 2000; Licois, 2002; Dewree et al., 2003; Marlier et al., 2003b; Licois et al., 2005), particularly in terms of contagiousness, rapid epizootic spread, persistence, clinical signs, pathology, immunosuppression, ineffective response to treatments, and high mortality. However, in ERE, no lesions were detected in liver, spleen, mesenteric ganglia, thymus, heart, kidneys, suprarenal gland (Licois et al., 2000), whereas in the present report lesions in these organs were present, probably due to differences in number of opportunistic pathogen agents implicated.

Mycosis and acid–alcohol resistant bacteria in lung tissue were not observed. Interstitial pneumonia was found in 57.1% of examined rabbits but the reason for this lesion is unknown. This proportion is similar to the 48% reported by Ventura et al. (2004) in examined rabbits affected with severe cases diarrhoeas in Mexico. Interstitial pneumonia in rabbits affected with ERE in Europe have been reported and are probably associated with viral infection (Licois, 1998). However, Licois et al. (2000) maintain that this lesion should be interpreted with care since lesions of the same type have also been found in healthy control rabbits. To ascertain for sure whether the disease present in Mexico is the same as that described in Europe opens an intriguing and challenging field of research, especially if we consider that in Europe efforts to find the virus pathogen as the main cause of ERE, responsible have not been conclusive (Legall et al., 1998; Licois et al., 2000; Licois et al., 2005).

More studies are necessary to determine the course of the disease and to identify the aetiological agents involved. Future investigations should gather laboratory examinations from different affected farms with a greater number of animals. A better understanding of the spontaneous evolution, pathology, and pathobiology of the disease would allow for the establishment of adequate programs of biosecurity, management, immunostimulation, vaccination, prophylaxis, sanitation, and nutrition practices in order to exert some control on this major rabbit emergent disease in Mexico and reduce economic losses.

Acknowledgements

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