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CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* PRESENCE AND CONCENTRATION OF ITS α-TOXIN IN THE CAECAL CONTENTS OF FATTENING RABBITS SUFFERING FROM DIGESTIVE DISEASES

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ABSTRACT: Digestive diseases are the main cause of morbidity and mortality in growing rabbits. *Clostridium perfringens* is a widely occurring pathogenic bacterium in enteric diseases of domestic animals and its pathogenicity stems from the production of potent exotoxins. This work aimed to quantify the concentration of *C. perfringens* α-toxin in caecal samples of rabbits with digestive diseases and relate these concentrations to *C. perfringens* counts. Additionally, *C. perfringens* strains isolated from rabbits with clinical lesions of Epizootic Rabbit Enteropathy (ERE) were toxinotyped. To conduct this work, a total of 711 rabbits weaned at 35 d were housed in pairs and fattened until the age of 63 d. No experimental infection was performed and no antibiotics were provided in the feed or drinking water. All rabbits displaying symptoms of digestive diseases were slaughtered and necropsied. At 46 d, 88 healthy rabbits were also slaughtered. Caecal contents were sampled from all slaughtered animals. Thirty-seven out of the 69 rabbits with digestive diseases (88.5% of sick animals) showed ERE-confirmed symptoms and lesions. Apart from diarrhoea, the most constant ERE signs were abdominal bloating and borborygmi. At necropsy, the anterior digestive tract was found filled with large amounts of gas and liquid. Twenty-seven rabbits had liquid caecal contents whereas caecal impaction only appeared in 10 rabbits. Live weight was lower by 49.2% (*P*<0.001) in diseased rabbits as compared with healthy rabbits of the same age. For *C. perfringens* counts lower than 6.0 log cfu/g, the α-toxin concentration remained below 2.6 μg/mg. However, for bacterial counts above 6.0 log cfu/g the concentration of α-toxin ranged from 0.12 to 60.9 μg/mg. Nevertheless, both caecal concentration of *C. perfringens* (7.65 *vs*. 3.09 log cfu/g, *P*<0.001) and that of its α-toxin (6.02 *vs*. 0.17 μg/mg, *P*<0.001) were higher in diseased rabbits than in healthy ones. *C. perfringens* toxinotype A was found in all ERE-affected rabbits. No other toxinotype was identified and no isolate contained the enterotoxin gene. In conclusion, *C. perfringens* α-toxin should not be considered a good indicator of the bacterium's presence, as high counts of colonies are not always associated with high toxin concentrations.

Key Words: Alpha-toxin, *Clostridium perfringens*, digestive diseases, Epizootic Rabbit Enteropathy, growth depression, toxinotype.

INTRODUCTION

Since 1996, Epizootic Rabbit Enteropathy (ERE) has spread very quickly throughout Europe, dramatically raising average mortality rates in industrial fattening rabbit farms (up to 70%, Boucher and Nouaille, 2002; Licois *et al.*, 2006; Romero *et al.*, 2010) and becoming one of the main causes of urgent visits by veterinary practitioners to commercial farms (Rosell *et al.*, 2009).

This enteropathy is a digestive disease characterised by prostration, abdominal bloating, aqueous diarrhoea and distension of both stomach and small intestine with gaseous and watery contents, both impacted or liquid caecal contents and occasionally droppings with abundant mucus in 10% of the cases (Coudert *et al.*, 1997; Licois *et al.*, 2006; Dewrée *et al.*, 2007). Although increased mortality rates mainly account for the economic losses caused by this disease, farm profitability is also affected by the impairment of growth performance and feed conversion (Licois, 2004, 2010). Indeed, ERE is responsible for sharp decreases in feed intake and anorexia (Boucher and Nouaille, 2002) so that surviving animals reach the slaughter age with lower body weight and lesser homogeneity (Licois, 2007; Romero *et al.*, 2009a).

Despite the detrimental effects of this disease, its aetiological agent remains unknown (Szalo *et al.*, 2007). Gram-positive bacterium *Clostridium perfringens* (*C. perfringens*) has been isolated at high counts in faecal samples of rabbits that died of ERE (Marlier *et al.*, 2006) and in the reference inoculum used to reproduce the pathology (Licois *et al.*, 2005; Huybens *et al.*, 2009). The role of this opportunistic bacterium is still under debate, even though it has been shown as an indicator of ERE prevalence (Romero *et al.*, 2009b). *C. perfringens* is a ubiquitous anaerobic spore-forming rod commonly found in the gut microbiota of animals and the environment (Petit *et al.*, 1999). Nevertheless, it is also an important pathogen, in humans and farming animals alike, as it causes gas gangrene, necrotic enteritis and foodborne illnesses (Rood, 1998). As *C. perfringens* does not invade healthy cells in normal conditions, its pathogenicity stems from the production of four major potent exotoxins (α, β, ε and ι; Songer, 1996). On the basis of the exotoxins produced, *C. perfringens* strains are classified into 5 toxinotypes (A, B, C, D and E; Petit *et al.*, 1999). Among these types, *C. perfringens* type A is the most common one and is responsible for necrotic enteritis in domestic avian species and gangrene in human beings. According to Cocchi *et al.* (2007), toxinotype A would be the dominant type in ERE cases too but the only other work that also typed *C. perfringens* in animals affected by ERE found a lower percentage of type A isolates (Marlier *et al.*, 2006). The only major exotoxin synthesised by type A strains is the α-toxin (Baums *et al.*, 2004), which is actually the principal lethal toxin and can be produced by all 5 types (Songer, 1996). Thus, the quantification of α-toxin could be used as a way to confirm the presence of *C. perfringens*. However, to the authors' knowledge, α-toxin has not hitherto been quantified in digestive samples of rabbits affected by ERE.

Therefore, this work aimed to quantify the concentration of α-toxin in caecal samples of rabbits with digestive diseases and relate these concentrations to *C. perfringens* counts. An additional objective was to genotype *C. perfringens* strains isolated from rabbits with ERE clinical lesions.

MATERIALS AND METHODS

Animals and housing

This study was approved by the Ethics Committee of the Universidad Politécnica de Madrid (Spain). Rabbits were handled according to the principles for the care of animals in experimentation (Spanish Royal Decree 1201/2005).

The study consisted of 2 fattening trials conducted at the experimental facilities of the University with 3-way crossbred rabbits originating from crossbred females (line V×line A) crossed with line R males. The latter lines were genetically improved at the Universitat Politècnica de València (Spain). In both trials, kits were weaned at 35 d of age (212 and 499 weanling rabbits in the 1st and 2nd trials, respectively) and fattened until the age of 63 d. The 1st and 2nd trial corresponded,

respectively, to the 1st and 4th parturition (theoretical parturition interval of 42 d) of the mothers of the rabbits used in this work. At weaning, rabbits were moved to a separate building and housed in pairs in flat-deck cages measuring 600×250×330 mm. Rabbits were always kept under controlled environmental conditions (room temperature between 16 and 24°C; with a 12-h photoperiod; light was switched on at 7:30).

Before the beginning of each trial, cages, walls, ceiling and floor were cleaned using high pressure water containing a detergent (RM806 ASF, Alfred KärcherGmbH & Co. KG) and disinfected spraying a product active against Gram-positive and Gram-negative bacteria, spores, virus, fungi, micoplasms, acari and insects (Sanivir Plus®, Bioplagen, S.L. containing: 15% glutaraldehyde, 10% didecilmethyl ammonium chloride, 10% cypermethrin, solvents and excipients). Five litres of a 2% Sanivir Plus® aqueous solution were sprayed to disinfect a surface of 100 m² (1 mL of Sanivir Plus®/m²).

Feeding

A common experimental diet meeting the essential nutrient requirements of fattening rabbits was used in both trials. The main ingredients of this diet were barley (24.0%), dehydrated alfalfa (28.0%), sunflower meal (19.9%) and beet pulp (15.0%). As a result of the chemical analyses performed, the diet was found to contain 42.4% neutral detergent fibre, 24.8% acid detergent fibre, 6.70% acid detergent lignin, 15.2% starch and 18.2% crude protein, on a dry matter (DM) basis. The digestible energy content of the diet was determined *in vivo* by Romero *et al.* (2011) and amounted to 10.8 MJ/kg DM. Animals always had *ad libitum* access to feed and water throughout the whole fattening period (35-63 d). With the exception of a coccidiostatic additive (60 ppm of robenidine), no other medications were provided in the feed or drinking water.

Health status evaluation

From weaning until the end of the fattening period (63 d), all rabbits were visually controlled twice a day for the appearance of symptoms such as prostration, bloated abdomen, diarrhoea or mucus under the cage. In both trials, all visual examinations of animals were performed by the same person. Complete and detailed descriptions of daily observations were recorded on previously designed and standardised tables. The morbidity rate was calculated as the percentage of live rabbits displaying some of these symptoms or all of them. At these inspections, the number of dead-found rabbits was also recorded. Every time the mentioned symptoms were detected, all morbid rabbits were weighed, slaughtered by cervical dislocation and an individual sample of caecal contents (5 g, approximately) was collected, immediately put in a sterile polystyrene tube and analysed subsequently for enumeration of *C. perfringens*. Afterwards, these caecal samples were frozen and retained for later quantification of the concentration of *C. perfringens* α-toxin. Every morbid animal was necropsied and macroscopic symptoms observed in the gastrointestinal tract were recorded (special attention was given to the aspect of the walls of the different parts of the digestive system, to the contents of the stomach, ileum and caecum and to the appearance and degree of diarrhoeas as well as the presence of mucus in droppings). A rabbit with mild diarrhoea, distension of the anterior digestive tract with gas and liquid contents, liquid or impacted caecal contents and without macroscopic signs of inflammation was deemed as an ERE-confirmed case. When clinical signs and lesions of ERE were identified, the toxinotype of *C. perfringens* (Petit *et al.*, 1999) was determined by PCR using primer sequences of Baums *et al.* (2004) and Gurjar *et al.* (2007). Rabbits were naturally infected. No experimental infection was done.

Eighty-eight healthy rabbits originating from both trials (n=44 per trial) were slaughtered in a hermetically-closed CO_2 chamber at 46 d of age. Caecal contents of all these 88 animals were sampled and tested for count of *C. perfringens*. Caecal concentration of the α-toxin was determined in 48 (n=24 per trial) out of the 88 rabbits. These concentrations were used as a reference of healthy animals.

In the 2nd trial, a different group of 100 healthy rabbits randomly chosen was constituted at weaning. These rabbits were weighed on 35 (weaning), 38, 42, 45, 49, 52, 56, 59 and 63 d of age. At each age, the average weight was determined. The 9 means were used to plot a reference growth curve of healthy rabbits.

Microbiological analyses

C. perfringens enumeration was performed according to Standard 7937 (ISO, 1997). This technique analyses all the *C. perfringens* toxinotypes. Sampling was achieved by blending each 1-g sample of digestive content with 9 mL of peptone water. All blended samples were vortexed and further diluted tenfold. All dilutions (8 dilutions per sample) were plated to determine the population of the suspensions. The culture medium was agar tryptose sulphite added with antibiotic D-cycloserine. Agar plates were incubated at 37°C in anaerobic jars for 18 h.

To quantify *C. perfringens* α-toxin in the frozen caecal contents, the Bio-X Alpha Toxin Elisa kit (Bio-X Diagnostics, Jemelle, Belgium) was used following the procedure outlined in the manufacturer manual (Bio-X Diagnostics BIO K084). All caecal content samples collected during the fattening period were thawed at the end of each trial and analysed within 2 d. A standard curve was built with the corresponding net absorbance values of tenfold serial dilutions of the α-toxin reference antigen. Dilutions were made with the dilution buffer provided in the kit. Absorbance was measured at 450 nm using a plate reader. The net absorbance readings were obtained by subtracting the absorbance of the blanks (wells with non-specific antibodies) from the absorbance of the samples. All samples and serial dilutions of the standard were analysed in duplicate with their corresponding blanks. Finally, the α-toxin concentration in samples was calculated using the standard curve equation (Zhang *et al.*, 2006).

Clostridium perfringens genotyping

From each slaughtered rabbit affected by supposed ERE, another fresh sample (1 g, approximately) of caecal contents was collected in a sterilised plastic tube, immediately placed on ice and stored at –80°C for further analysis. The QIAamp DNA Stool Mini KitTM (Qiagen, CA, USA) was used to isolate bacterial DNA with a previous sample treatment with reinforced *Clostridium* medium following the method used by Gurjar *et al.* (2007). Three independent duplex PCR reactions were standardised for the detection of *Clostridium perfringens* genes encoding α-, β-, ε-, ι-, β2- and CPE toxins with available specific primer sequences and conditions published by Baums *et al.* (2004) and Gurjar *et al.* (2007). Detailed information regarding duplex PCR conditions can be found in Menoyo *et al.* (2011).

Chemical analyses

Chemical analyses of the experimental diet were conducted in triplicate. AOAC procedures (2000) were used to determine DM by the oven-drying method (930.15), total ash by muffle furnace (942.05), Dumas N (968.06) using a LECO equipment (model FP-528, Leco Corporation, St. Joseph, MI, USA) and starch (996.11). NDF content was determined using the Mertens method

(2002), whereas the analyses of ADF and ADL were done according to the official method (973.18) of the AOAC (2000). All fibre analyses were done sequentially and corrected by ash content of ADL residue.

Statistical analysis

All data were analysed for normal distribution using the UNIVARIATE procedure and for homogeneity of variances through the Levene's Test using the HOVTEST option of GLM procedure of SAS (1990). The experimental unit was the rabbit in all analyses.

C. perfringens counts were analysed using generalised linear models (McCullagh and Nelder, 1989) with the GENMOD procedure of SAS (1990). A Poisson distribution was used for the *C. perfringens* caecal counts and the link function was the natural logarithm, which related the mean count with the linear combination of the explanatory variables used in these analyses. The explanatory variables were the trial, the symptoms and their interaction.

Results from α-toxin concentration were analysed as a completely randomised design with the trial, the symptoms and their interaction as the main sources of variation by using the GLM procedure from SAS (1990).

These procedures were also used to compare *C. perfringens* caecal counts and the concentration of α-toxin in healthy and diseased rabbits.

The correlation coefficient between the *C. perfringens* count and the concentration of α-toxin in caecal contents was obtained with the CORR procedure by SAS (1990). Regression procedures (REG procedure, SAS, 1990) were used to obtain the equation of the reference growth curve.

RESULTS AND DISCUSSION

All rabbits displaying clinical symptoms of digestive diseases were slaughtered upon detection of the symptoms. In total, 31 and 38 morbid rabbits were slaughtered and necropsied in the 1st and 2nd trial, respectively. Thus, the morbidity rate could be calculated (14.6 and 7.61%). In both trials some rabbits died of causes other than digestive diseases (pasteurellosis and respiratory diseases) or were culled due to extremely low body weight. The number of dead-found animals amounted to 3 and 6 in the 1st and 2nd trial, respectively. If considering that morbid animals would have eventually died, the global mortality rate could also be calculated for both trials (16.0 and 8.82%, respectively). On balance, rabbits with digestive diseases represented 88.5% of sick animals. Accordingly, Licois (2004) and Boucher and Leplat (2005) stated that digestive diseases are the main cause of morbidity and mortality in growing rabbits.

Thirty-seven out of the 69 animals with digestive diseases showed ERE symptoms (Licois *et al.*, 2006; Dewrée *et al.*, 2007; Licois, 2010). Bar one rabbit, all animals affected by ERE presented diarrhoea. However, it is worth noting that in almost all animals diarrhoeas were of weak intensity as informed by Boucher and Nouaille (2002). According to Licois (2004, 2010), diarrhoea is the most dominant clinical sign of ERE as it can be found in more than 95% of affected rabbits. Apart from diarrhoea, the most constant clinical signs were abdominal bloating and gurgling, which are not pathognomonic symptoms. At necropsy, these signs were confirmed by the gross lesions observed. Affected rabbits were bloated because of distension of stomach and small intestine. It was repeatedly found that the anterior digestive tract was filled with large amounts of gas and watery contents. These gross lesions were common features in rabbits developing ERE.

In some cases, gas accumulation in the caecum was also observed. In agreement with previous descriptions of ERE symptoms (Licois *et al.*, 2005; Maertens *et al.*, 2005; Licois, 2010), no visible inflammation of the walls of the intestinal tract was detected in ERE-affected animals. It was also observed that the distal colon was often empty or just contained mucus.

At necropsy, it was also recorded that 27 rabbits had liquid caecal contents whereas caecal impaction only appeared in the other 10 rabbits with ERE symptoms. Licois *et al.* (2005) also found that cases of impacted caecal contents were less frequent (20 to 30% of the animals). Besides, caecal impaction is deemed a final step in paresia of the digestive tract, while rabbits in this study were slaughtered as soon as clinical signs were detected. In the present work, mucoid diarrhoea was observed in 12 rabbits affected by ERE. Nine cases corresponded to animals having liquid caecal contents. Although mucus excretion is common to several diseases (Licois *et al.*, 2006), Coudert *et al.* (1997) and Boucher and Nouaille (2002) noted at least 10% of the rabbits suffering from ERE usually present mucoid diarrhoeas. Furthermore, these authors highlighted the very abundant presence of mucus in some affected rabbits.

One of the first signs observed in diseased rabbits was the low body weight. Figure 1 presents the growth curve of 100 healthy rabbits and the weight at slaughter of all morbid animals (either confirmed ERE or non-specific diarrhoeal syndromes). On average, the live weight was lower by 49.2% ($P<0.001$) in the diseased rabbits as compared with the corresponding weight of healthy rabbits of the same age. Growth depression in rabbits affected by ERE has consistently been mentioned in previous reports (Boucher and Nouaille, 2002; Coudert and Licois, 2005; Maertens *et al.*, 2005; Licois *et al.*, 2006; Licois, 2007; Romero *et al.*, 2009a). Coudert and Licois (2005) were even unable to avoid the fall in the growth rate of rabbits treated with bacitracin. In the study by Licois *et al.* (2005), daily weight gain of control rabbits was also sharply decreased when they were housed in the same room as the inoculated groups. Finally, Licois (2004) surmised that the growth depression and impaired feed conversion could lead to greater economic losses than mortality.
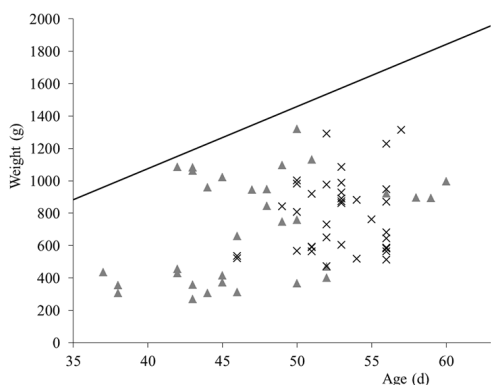

Figure 1: Growth curve of healthy growing rabbits and weight of morbid rabbits with digestive diseases. (——) Growth curve of healthy rabbits, (▲) Sick rabbits with non-specific diarrhoeas, (×) Rabbits affected by ERE. (Weight=38.40×Age–459; n=9 means of 100 rabbits; RSD=71.1; R^2=0.97; $P<0.001$).

With respect to the total number of morbid rabbits, the percentage of animals affected by ERE was 53.6% (Table 1). Even if this percentage could be in keeping with that (52.9%) found by Maertens *et al.* (2005), the ERE morbidity seems very low. ERE is characterised by spreading very quickly and morbidity can rise to 100% after experimental inoculation (Licois *et al.*, 2005). Nonetheless, it is true that the overall morbidity in the current trials was rather low (9.70%), especially taking into account that no antibiotics were provided in the feed or drinking water (Licois *et al.*, 2005; Maertens *et al.*, 2005; Romero *et al.*, 2010). This good health status is likely due to the hygienic and zootechnical practices applied at the farm (Garrido *et al.*, 2009; Romero *et al.*, 2009a).

Table 1: Characterization of *C. perfringens* presence in ERE[1]-confirmed cases.

Age (d)	Weight (g)	Caecal concentration of *C. perfringens* (log cfu/g)	Caecal concentration of α-toxin (µg/mg)	*C. perfringens* toxinotype
Trial 1				
50	567	7.00	2.14	A
51	592	6.90	4.82	A
52	729	7.08	16.2	A
52	471	4.60	undetected	A
52	650	8.00	60.9	A
53	926	6.90	0.25	A
53	861	7.04	undetected	A
54	517	9.00	11.2	A
56	582	8.00	0.41	A
56	644	6.70	1.49	A
56	587	4.90	0.16	A
56	583	4.48	0.52	A
56	564	5.48	undetected	A
56	513	8.00	27.1	A
56	948	6.90	0.84	A
Trial 2				
46	535	8.00	6.35	A
46	521	7.48	9.75	A
49	841	7.86	11.2	A
50	1003	6.56	5.64	A
50	982	7.38	25.3	A
50	807	6.95	38.9	A
51	919	6.41	4.25	A
51	590	8.18	4.25	A
51	565	8.00	undetected	A
52	1292	8.18	16.8	A
52	976	7.40	11.2	A
53	987	6.79	9.75	A
53	1085	7.90	2.42	A
53	873	7.60	11.3	A
53	889	8.04	0.16	A
53	603	8.15	0.52	A
54	881	7.26	3.31	A
55	761	6.93	0.52	A
56	871	6.66	5.24	A
56	1227	8.30	undetected	A
56	682	7.95	2.14	A
57	1315	6.85	0.71	A

[1] Epizootic Rabbit Enteropathy.

Tables 2a and 2b describe the clinical symptoms observed in rabbits suffering from digestive disorders that were not confirmed as ERE. Although various rabbits presented anorexia, none of them displayed noticeable abdominal distension. Besides, some of the lesions considered as

Table 2a: Trial 1: Characterization of *C. perfringens* presence in animals showing non-specific diarrhoeal symptoms.

Age (d)	Weight (g)	Caecal concentration of *C. perfringens* (log cfu/g)	Caecal concentration of α-toxin (μg/mg)	Symptoms
37	435	4.18	undetected	diarrhoea, anorexia, impacted dry gastric contents
38	307	3.23	0.71	abundant diarrhoea, anorexia, gas in stomach and caecum
38	355	2.48	0.97	diarrhoea, low body weight
42	454	2.90	0.08	diarrhoea, anorexia, gastrointestinal dilation
42	429	3.36	0.30	diarrhoea, low body weight, gas in caecum
43	358	3.54	2.57	diarrhoea, low body weight
43	268	3.04	0.20	anorexia, abundant diarrhoea, small intestine dilation
44	306	3.00	1.19	abundant diarrhoea and low body weight
45	416	3.53	0.12	diarrhoea but no other apparent symptoms
45	372	2.95	undetected	abundant diarrhoea, gas in ileum and caecum
46	657	3.40	undetected	diarrhoea, acute ileum dilation, bloody intestinal contents
46	313	3.95	0.05	diarrhoea, low body weight
50	367	3.75	2.14	low body weight, diarrhoea, gas in stomach and ileum
50	757	4.08	0.18	diarrhoea, liquid caecal contents, marked vascularisation
52	401	2.30	undetected	abundant diarrhoea with mucus, marked vascularisation in small intestine, gas in caecum
52	468	4.18	0.02	aqueous diarrhoea with mucus

being pathognomonic of ERE were not found in these rabbits. It is true that gas was found in stomach and small intestine in numerous rabbits, but no physiopathological alterations typical of ERE were revealed, as the anterior digestive tract was not overflowing with alarming amounts of gas or liquid and the aspect of caecal contents was apparently that of healthy rabbits. However, it should be pointed out that it is sometimes difficult to diagnose ERE with absolute certainty in the field. ERE is indeed a non-specific enteropathy (Maertens *et al.*, 2005) and the criteria used to distinguish it from other intestinal disorders lack specificity (Licois *et al.*, 2006). In fact, the distension of stomach and small intestine by a very liquid and gaseous content that entails the abdominal bloating is probably the only distinctive feature of ERE along with the absence of macroscopic signs of inflammation (Licois *et al.*, 2005; Licois, 2010). Therefore, it could be thought that several of the rabbits classified as showing non-specific diarrhoeal symptoms were actually also suffering from ERE but at an early stage of the disease. These rabbits were slaughtered on average 12.0±5.73 d after weaning, whereas slaughter of ERE-confirmed animals occurred on average 17.8±2.76 d after weaning. In some cases, these non evident symptoms were perhaps a prelude to the complete development of clearer ERE signs. Moreover, previous research reported that there is usually a peak in the number of ERE deaths around 15 d after weaning (Boucher and Nouaille, 2002; Maertens *et al.*, 2005; Garrido *et al.*, 2009; Romero *et al.*, 2009a,b).

Table 2b: Trial 2: Characterization of *C. perfringens* presence in animals showing non-specific diarrhoeal symptoms.

Age (d)	Weight (g)	Caecal concentration of *C. perfringens* (log cfu/g)	Caecal concentration of α-toxin (μg/mg)	Symptoms
42	1086	2.70	undetected	diarrhoea, gas in stomach and ileum
43	1081	3.70	0.16	diarrhoea, gaseous dilation of jejunum
43	1062	2.00	undetected	diarrhoea, gas in stomach and ileum
44	960	2.18	0.12	diarrhoea, gas in ileum
45	1023	4.60	2.48	diarrhoea, gas in ileum
47	945	4.00	0.12	diarrhoea, impacted dry gastric contents, gas in ileum
48	946	2.00	undetected	diarrhoea, gas in stomach and ileum
48	845	2.00	0.46	diarrhoea, gas in stomach and ileum
49	1096	5.51	0.90	diarrhoea, marked vascularisation in small intestine, gas in stomach and acute gaseous dilation of ileum
49	748	2.00	undetected	diarrhoea, liquid gastric and caecal contents
50	1320	7.00	0.64	diarrhoea, gas in stomach and ileum
51	1131	8.06	undetected	diarrhoea, liquid gastric contents, gas in ileum
56	922	8.11	0.30	aqueous diarrhoea with mucus, gas in stomach and ileum
58	895	7.90	15.6	diarrhoea, droppings with abundant mucus
59	894	7.54	0.12	aqueous diarrhoea with mucus, gas in stomach and ileum
60	995	4.70	undetected	diarrhoea, gas in stomach

Otherwise, some symptoms presented in Tables 2a and 2b such as bloody intestinal contents, abundant diarrhoea or impacted dry gastric contents are definitively not considered typical ERE lesions (Coudert *et al.*, 2000; Licois, 2010). Furthermore, Table 3 adds microbiological differences between rabbits in the present study. In rabbits displaying ERE-confirmed signs, the caecal concentration of *C. perfringens* (7.88 *vs.* 5.51 log cfu/g, $P<0.001$) and that of its α-toxin (9.49 *vs.* 1.40 μg/mg, $P=0.008$) were higher than in rabbits showing non-specific symptoms. When comparing all diseased rabbits to healthy ones, both the caecal concentration of *C. perfringens* (7.65 *vs.* 3.09 log cfu/g, $P<0.001$) and that of its α-toxin (6.02 *vs.* 0.17 μg/mg, $P<0.001$) were higher in diseased rabbits. *C. perfringens* is undoubtedly an important pathogen that causes gas gangrene and food poisoning in human beings, necrotic enteritis in fowl and enterotoxaemia in young ruminants (Songer, 1996; Rood, 1998; Petit *et al.*, 1999). Elevated counts (4 to 7 log cfu/g) are often found in the intestinal contents of lambs suffering from enterotoxaemia (Songer, 1996). Marlier *et al.* (2006) isolated *C. perfringens* in more than 80% of rabbits affected by ERE while Licois *et al.* (2005) and Huybens *et al.* (2009) identified it in the reference inoculum used to reproduce the disease. Additionally, in 83% of the rabbits affected by ERE in which Marlier *et al.* (2006) managed to isolate *C. perfringens*, faecal counts were above 4 log cfu/g. Furthermore, bacitracin, which acts on Gram-positive bacteria like *C. perfringens*, has been shown effective to control ERE mortality (Duperray *et al.*, 2003;

Table 3: Caecal concentration of *C. perfringens* and that of its α-toxin in healthy and diseased rabbits in two fattening trials.

		Caecal concentration of *C. perfringens* (log cfu/g)		Caecal concentration of α-toxin (μg/mg)	
Diseased rabbits[1]		No.		No.	
Trial	1	31	7.01	24	5.61
	2	38	7.58	30	5.29
Symptoms[1]	ERE	37	7.88	32	9.49
	Non-specific	32	5.51	22	1.40
			-		RSD=10.5 (n=27)
P trial			0.37		0.91
P symptoms			<0.001		0.008
P trial×symptoms			0.20		0.57
Diseased *vs.* healthy rabbits		No.		No.	
Diseased rabbits[1]		69	7.65	54	6.02
Healthy rabbits		88	3.09	48	0.17
			-		RSD=7.96 (n=51)
P-value			<0.001		<0.001

[1] Digestive diseases: either symptoms of Epizootic Rabbit Enteropathy or non-specific diarrhoeal symptoms.

Maertens *et al.*, 2005; Richez *et al.*, 2007). Based on all these facts, some authors (Licois, 2004; Romero *et al.*, 2009b) suspected that *C. perfringens* was implicated in the development of ERE. However, its role as pathogenic agent remains questionable since it has not been possible to experimentally reproduce ERE with a pool including strains of *C. perfringens* and other Gram-positive bacteria (Marlier *et al.*, 2006). Even though *C. perfringens* does not seem to be the primary cause of ERE, this bacterium is likely to play a role in the soaring of mortality rates (Licois, 2010). Consequently, it has been hypothesised that *C. perfringens* might be an opportunistic bacterium proliferating in a gut dysbiosis, especially as a post-mortem invader in dead-found animals (Songer, 1996). Nevertheless, it should be recalled that rabbits in the present study were slaughtered upon detection of the symptoms, so that samples did not originate from dead animals. Whichever the hypothesis considered, a more recent work continues to suggest that a bacterium and a toxin seem involved in the pathogenesis of ERE (Huybens *et al.*, 2009). *C. perfringens* α-toxin, which is produced by the 5 toxinotypes (Petit *et al.*, 1999), is a potently lethal dermonecrotic toxin and plays an essential role in the pathogenesis of human gas gangrene (Rood, 1998). Some of the lesions observed in this work could in part reflect the effects of the α-toxin, which are mainly gas production and tissue damage. In fact, this toxin hydrolyses lecithin, phospholipids and sphingomyelin and causes membrane disruption (Songer, 1996). In this scope, Marlier *et al.* (2006) established a correlation (*P*<0.001) between the appearance of ERE typical gross lesions and the presence of the *C. perfringens* α-toxin. However, it should be acknowledged that in this work some of rabbits for which the α-toxin was not detected presented similar lesions to those with high concentrations of α-toxin. Moreover, Timbermont *et al.* (2009) reported that the ability to induce necrotic lesions in chickens was independent of the capacity of *C. perfringens* strains to synthesise α-toxin.

Figure 2 shows the α-toxin concentration in relation to *C. perfringens* count. It can be seen that for all counts lower than 6.0 log cfu/g, the α-toxin concentration remained below 2.6 μg/mg.

However, bacterial counts above 6.0 log cfu/g did not necessarily imply high concentrations of α-toxin, as values ranged from 0.12 to 60.9 µg/mg. *C. perfringens* is a normal inhabitant of the gastrointestinal tract of animals but under some predisposing environmental and nutritional factors (Timbermont *et al.*, 2009), the number of vegetative cells can rise up to high counts with a relatively fast growth rate. The exponential growth is accompanied by the secretion into the medium of the toxins (Rood, 1998; Petit *et al.*, 1999). This could justify why a high count of vegetative cells of *C. perfringens* is required to find a high concentration of α-toxin. However, it seems not to be a sufficient condition. A great variability in the α-toxin producing capacity of *C. perfringens* strains has been demonstrated in previous research (Möllby *et al.*, 1976; Liu and Blaschek, 1996; Hale and Stiles, 1999),

Figure 2: α-toxin concentration (µg/mg) in relation to *C. perfringens* count (log cfu/g) in the caecal contents of young fattening rabbits (n=102, 54 diseased rabbits+48 healthy rabbits).

even when bacteria belonged to the same toxinotype (Timbermont *et al.*, 2009). Moreover, in 15 diseased rabbits of this work, it was not possible to quantify the α-toxin, regardless of the *C. perfringens* count (Tables 1 and 2a,b). Eleven out of these 15 rabbits had caecal concentrations of *C. perfringens* below the threshold of 6.0 log cfu/g (3.30 log cfu/g, on average). Likewise, Marlier *et al.* (2006) failed to detect the presence of the α-toxin in the intestinal contents of 31.2 and 83.3% of the rabbits that had died of ERE or other digestive disorders, respectively. These negative results are certainly due to the fact that the α-toxin concentration was under the detection limit of the assay. We doubt that mutants defective in α-toxin production were present in the caecal samples. As a final result, the correlation coefficient between the count of *C. perfringens* and the concentration of α-toxin in all caecal contents was r=0.49 (*P*<0.001; n=102, 54 diseased rabbits+48 healthy rabbits).
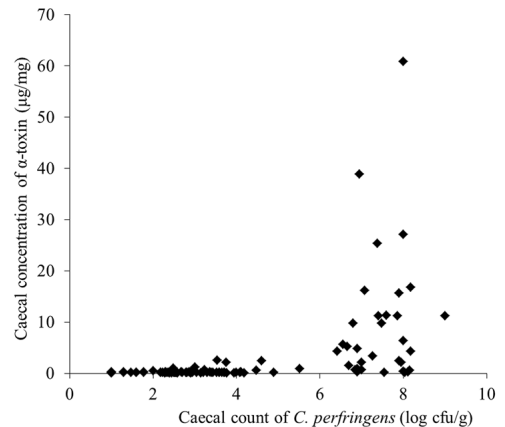
C. perfringens was isolated from all examined rabbits affected by ERE. Results of PCR analysis showed that all 37 animals affected by ERE were positive only for the alpha toxin gene (α), being hence classified as toxinotype A. This result is consistent with that of Cocchi *et al.* (2007) who found that 99% of the isolates sampled from rabbits affected by enteropathy belonged to toxinotype A. In the study by Marlier *et al.* (2006), ⅔ of the isolates originating from rabbits that had died of ERE were of toxinotype A and the other third of toxinotype C. In the current work, no other toxinotype was identified apart from type A and none of the isolates contained the enterotoxin gene nor was positive for the β2 gene. Marlier *et al.* (2006) only found the β2 gene in one out of the 62 isolates typed, whereas Cocchi *et al.* (2007) evidenced its presence in 25% of the strains. Licois *et al.* (2006) remarked that αβ2 strains were frequently found in rabbits with impacted caecal contents. However, the 10 rabbits in this work presenting caecal impaction were all of toxinotype A. In other species, toxinotype A of *C. perfringens* was detected in 64% of sheep with enterotoxaemia (Kalender *et al.*, 2005) and in 100% of piglets suffering from enteritis (Wasinski, 2007). As reviewed by Songer (1996) and Petit *et al.* (1999), strains of toxinotype A are the most common in the intestines. Nevertheless, before the spread of ERE, toxinotype E was much more frequently isolated in diseased rabbits (Patton *et al.*, 1978; Baskerville *et al.*, 1980).

Although there is nowadays a clear prevalence of genotype α, both α-β2 and α-cpe genotypes have also been detected in isolates from ERE-affected rabbits (Licois *et al.*, 2006; Menoyo *et al.*, 2011). The presence of cpe gene in strains of toxinotype A is closely linked to the onset of diarrhoeic conditions in domestic animals (Songer, 1996), so further research may focus on the involvement of α, β2 and CPE toxins in toxinotype A strains of *C. perfringens* isolated from rabbits affected by ERE.

CONCLUSIONS

Clostridium perfringens α-toxin should not be considered a good indicator of bacterium presence, as high counts of vegetative cells are not always associated with high concentrations of the toxin. However, rabbits with digestive diseases presented high caecal counts of both *C. perfringens* and its α-toxin. All *C. perfringens* strains isolated from rabbits with ERE lesions belonged to toxinotype A.

REFERENCES

AOAC. 2000. Official Methods of Analysis, *17th Ed. Association of Official Analytical Chemists, Arlington, VA.*

Baskerville M., Wood M., Seamer J.H. 1980. *Clostridium perfringens* type E enterotoxaemia in rabbits. *Vet. Rec., 107: 18-19. doi: 10.1136/vr.107.1.18*

Baums C.G., Schotte U., Amtsberg G., Goethe R. 2004. Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. *Vet. Microbiol., 100: 11-16. doi: 10.1016/S0378-1135(03)00126-3*

Bio-X Diagnostics BIO K084 (currently BIO K289). *Clostridium perfringens* Alpha toxin antigen detection kit. Bio-X Diagnostics, Jemelle, Belgium. *Available at: http://www.biox.com/Default.aspx?tabid=64&udtid=201 Accessed September 2011.*

Boucher S., Leplat A. 2005. De quoi meurent les lapins d'engraissement? Table Ronde: Effets des conduites post-sevrage sur les performances et la santé des lapereaux. *Proc. In: 11èmes Journées de la Recherche Cunicole, Paris, France, Available at: http://www.asfc-lapin.com/Docs/Activite/T-ronde-2005/ Tables-rondes-01a.htm#aliment Accessed August 2011.*

Boucher S., Nouaille L. 2002. Manuel pratique: Maladies des lapins. *Éditions France Agricole, Second Edition, France.*

Cocchi M., Agnoletti F., Bacchin C., Bano L., Guolo A., Drigo I. 2007. Tossinotipizzazione di *Clostridium perfringens* isolato da coniglio. *In Proc.: Giornate di Coniglicoltura ASIC, Forlì, Italy, 67-69.*

Coudert P., Licois D. 2005. Epizootic rabbit enteropathy. Study of early phenomena with fresh inoculum and attempt at inactivation. *World Rabbit Sci., 13: 229-238.*

Coudert P., Lebas F., Licois D. 1997. Une nouvelle pathologie ravage les élevages. La profession se mobilise. *Cuniculture, 24: 225-229.*

Coudert P., Peeters J.E., Rosell J.M., Argüello J.L., Badiola J.I., Blas E., Cuervo L., Drouet-Viard F., Licois D., Provôt F. 2000. Enfermedades del aparato digestivo: causas parasitarias y otras. *In: Rosell J.M. (ed), Enfermedades del Conejo. Mundi-Prensa, Spain, 219-263.*

Dewrée R., Meulemans L., Lassence C., Desmecht D., Ducatelle R., Mast J., Licois D., Vindevogel H., Marlier D. 2007. Experimentally induced epizootic rabbit enteropathy: clinical, histopathological, ultrastructural, bacteriological and haematological findings. *World Rabbit Sci., 15: 91-102.*

Duperray J., Boisot P., Guyonvarch A., Richard A. 2003. Persistance de l'efficacité de la bacitracine pour lutter contre l'entéropathie épizootique du lapin (EEL) après quatre années d'utilisation sur le terrain. *In Proc.: 10èmes Journées de la Recherche Cunicole, Paris, France, 271-274.*

Garrido S., Nicodemus N., García J., Chamorro S., De Blas J.C. 2009. Effect of breeding system and farm hygiene on performances of growing rabbits and lactating does over two reproductive cycles. *World Rabbit Sci., 17: 71-78.*

Gurjar A.A., Hedge N.V., Love B.C., Jayarao B.M. 2007. Real-time multiplex PCR assay for rapid detection and toxintyping of *Clostridium perfringens* toxin producing strain in faeces of dairy cattle. *Mol. Cell. Probe., 22: 90-95.*

Hale M.L., Stiles B.G. 1999. Detection of *Clostridium perfringens* alpha toxin using a capture antibody ELISA. *Toxicon, 37: 471-484.doi: 10.1016/S0041-0101(98)00179-2*

Huybens N., Houeix J., Licois D., Mainil J., Marlier D. 2009. Inoculation and bacterial analyses of fractions obtained from the reference inoculum TEC4 which experimentally reproduces epizootic rabbit enteropathy. *World Rabbit Sci., 17: 185-193.*

ISO. International Organization for Standardization. 1997. *Method ISO 7937. Available at: http://www.iso.org/iso/ Accessed August 2011.*

Kalender H., Ertas H.B., Cetinkaya B., Muz A., Arslan N., Kilic A. 2005. Typing of isolates of *Clostridium perfringens* from healthy and diseased sheep by multiplex PCR. *Vet. Med.-Czech, 50: 439-442.*

Licois D. 2004. Domestic rabbit enteropathies. *In Proc.: 8th World Rabbit Congress, Puebla, México, 385-403.*

Licois D. 2007. Étude *in vivo* de la fraction surnageante de l'inoculum TEC4, inoculum utilisé pour la reproduction expérimentale de l'Entéropathie Epizootique du Lapin. *In Proc.: 12èmes Journées de la Recherche Cunicole, Le Mans, France, 217-220.*

Licois D. 2010. Pathologie d'origine bactérienne et parasitaire chez le lapin: apports de la dernière décennie. *Cuniculture, 37: 35-49.*

Licois D., Wyers M., Coudert P. 2005. Epizootic Rabbit Enteropathy: experimental transmission and clinical characterization. *Vet. Res., 36: 601-613.doi: 10.1051/vetres:2005021*

Licois D., Coudert P., Marlier D. 2006. Epizootic rabbit enteropathy. *In: Maertens L., Coudert P. (eds.), Recent Advances in Rabbit Sciences. Institute for Agricultural and Fisheries Research, Melle, Belgium, 163-170.*

Liu Z.L., Blaschek H.P. 1996. Monoclonal antibody-based ELISA for detection of *Clostridium perfringens* alpha-toxin. *J. Food Protect., 59: 621-625.*

Maertens L., Cornez B., Vereecken M., Van Oye S. 2005. Efficacy study of soluble bacitracin (Bacivet S®) in a chronically infected epizootic rabbit enteropathy environment. *World Rabbit Sci., 13: 165-178.*

Marlier D., Dewrée R., Lassence C., Licois D., Mainil J., Coudert P., Meulemans L., Ducatelle R., Vindevogel H. 2006. Infectious agents associated with epizootic rabbit enteropathy: Isolation and attempts to reproduce the syndrome. *Vet. J., 172: 493-500. doi: 10.1016/j.tvjl.2005.07.011*

McCullagh P., Nelder J.A. 1989. Generalized Linear Models, Second Edition. *Chapman and Hall, New York, USA.*

Menoyo D., García C., Jarava M., De Blas C. 2011. Genotyping of *Clostridium perfringens* strains in rabbits affected by epizootic enteropathy. *In Proc.: XIV Jornadas sobre Producción Animal, AIDA-ITEA, Zaragoza, Spain, 863-865.*

Mertens D.R. 2002. Gravimetric determination of amylase-treated neutral detergent fibre in feeds with refluxing beakers or crucibles: collaborative study. *J. AOAC Int., 85: 1217-1240.*

Möllby R., Holme T., Nord C.E., Smyth C.J., Wadström T. 1976. Production of phospholipase C (alpha-toxin), haemolysins and lethal toxins by *Clostridium perfringens* types A to D. *J. Gen. Microbiol., 96: 137-144.*

Patton N.M., Holmes H.T., Riggs R.J., Cheeke P.R. 1978. Enterotoxemia in rabbits. *Lab. Anim. Sci., 28: 536-540.*

Petit L., Gibert M., Popoff M.R. 1999. *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol., 7: 104-110. doi: 10.1016/S0966-842X(98)01430-9*

Richez P., Richard A., Cornez B. 2007. Sensibilité, résistance et profil de bactéricidie de la bacitracine vis-à-vis de souches de *Clostridium perfringens* isolées lors de cas cliniques d'Entéropathie Epizootique du Lapin. *In Proc.: 12èmes Journées de la Recherche Cunicole, Le Mans, France, 209-212.*

Romero C., Nicodemus N., Chamorro S., Corujo A., Martínez-Morentin C.G., Margüenda I., de Blas C. 2009a. Efecto de la camada y las condiciones ambientales sobre la mortalidad de gazapos en periodos con distinta incidencia de enteropatía epizoótica del conejo. *In Proc.: XIII Jornadas sobre Producción Animal, AIDA-ITEA, Zaragoza, Spain, 191-193.*

Romero C., Nicodemus N., García-Rebollar P., García-Ruiz A.I., Ibáñez M.A., de Blas J.C. 2009b. Dietary level of fibre and age at weaning affect the proliferation of *Clostridium perfringens* in the caecum, the incidence of Epizootic Rabbit Enteropathy and the performance of fattening rabbits. *Anim. Feed Sci. Tech., 153: 131-140. doi: 10.1016/j.anifeedsci.2009.05.005*

Romero C., Rebollar P.G., Dal Bosco A., Castellini C., Cardinali R. 2010. Efecto de la inclusión de ácidos orgánicos de cadena corta sobre el crecimiento, la mortalidad y el tejido linfoide del intestino en gazapos. *In Proc.: XXXV Symposium de Cunicultura, Segovia, Spain, 79-83.*

Romero C., Nicodemus N., Rodríguez J.D., García A.I., de Blas C. 2011. Effect of type of grinding of barley and dehydrated alfalfa on performance, digestion, and crude mucin ileal concentration in growing rabbits. *J. Anim. Sci., 89: 2472-2484. doi: 10.2527/jas.2010-3226*

Rood J.I. 1998. Virulence genes of *Clostridium perfringens*. *Annu. Rev. Microbiol., 52: 333-360. doi: 10.1146/annurev.micro.52.1.333*

Rosell J.M., de la Fuente L.F., Badiola J.I., Fernández de Luco D., Casal J., Saco M. 2009. Study of urgent visits to commercial rabbit farms in Spain and Portugal during 1997-2007. *World Rabbit Sci., 17: 127-136.*

SAS. 1990. SAS/STAT® *User's Guide, Version 6, 4th ed. SAS Inst., Cary, NC, USA.*

Songer J.G. 1996. Clostridial Enteric Diseases of Domestic Animals. *Clin. Microbiol. Rev., 9: 216-234.*

Spanish Royal Decree 1201/2005. 2005. Real Decreto 1201/2005. Sobre protección de los animales utilizados para experimentación y otros fines científicos. *Boletín Oficial del Estado, 252: 34367-34391.*

Szalo I.M., Lassence C., Licois D., Coudert P., Poulipoulis A., Vindevogel H., Marlier D. 2007. Fractionation of the reference inoculum of epizootic rabbit enteropathy in discontinuous sucrose gradient identifies aetiological agents in high density fractions. *Vet. J., 173: 652-657.doi: 10.1016/j.tvjl.2005.12.013*

Timbermont L., Lanckriet A., Gholamiandehkordi A.R., Pasmans F., Martel A., Haesebrouck F., Ducatelle R., Van Immerseel F. 2009. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. *Comp. Immunol. Microb., 32: 503-512.doi: 10.1016/j.cimid.2008.07.001*

Wasinski B. 2007. Contribution of *Clostridium perfringens* type A with β2 toxin gene in aetiology of porcine enteric diseases. A case report. *B. Vet. I. Pulawy, 51: 509-513.*

Zhang G., Darius S., Smith S.R., Ritchie S.J. 2006. *In vitro* inhibitory effect of hen egg white lysozyme on *Clostridium perfringens* type A associated with broiler necrotic enteritis and its α-toxin production. *Lett. Appl. Microbiol., 42: 138-143. doi: 10.1111/j.1472-765X.2005.01812.x*