World Rabbit Science

World Rabbit Sci. 2010, 18: 187 - 198
doi:10.4995/wrs.2010.775

CLINICAL, PATHOLOGICAL AND MICROBIOLOGICAL PROFILES OF SPONTANEOUS ENTEROPATHIES IN GROWING RABBITS

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Abstract: In a rabbit production facility, health monitoring for enteropathies was performed in 15 production cycles for 20 mo. For each cycle, up to a hundred 35 d old rabbits weaned the same day were randomly selected, reared in the same fattening unit, but separately from the source batch and fed with the same feed except for antimicrobial supplementation. Clinical symptoms and enteric lesions of the selected group were recorded, using two checklists with binomial response (yes/no answer to a list of 54 clinical and enteric variables). The day after weaning, one week later, at the beginning of the enteric symptoms and 4-5 d after the start of the symptoms, inocula from the small intestine and caecum of selected animals were subjected to microbiological, *C. spiroforme*, *Eimeria* oocyst and rotavirus antigen detection tests. Representative samples of *E. coli* and *C. perfringens* isolates were tested, respectively, for serotype, biotype, *eae*, *afr/2* genes and for α, β1, β2, ε, ι and enterotoxin toxin genes. The answers to the clinical-pathological variables were subjected to statistical analysis with a cluster analysis programme in order to obtain homogeneous, statistically significant groups of diseased animals (clusters). Then, the clusters were statistically associated with the laboratory outcomes. The cluster to which the enterotyphlitis lesions significantly contributed was associated with *E. coli* detection, *E. coli* O103 serotype detection and *C. spiroforme* ("several elements" variable). *C. spiroforme* ("rare elements" variable) was significantly associated with a cluster, characterised by a pathological profile consisting of bloating/rumbling noise and liquid content in stomach and caecum, without enteric inflammation. *C. perfringens* was significantly associated with a cluster, characterised by a pathological profile consisting of dilation/liquid content of small intestine, caecal impaction and mucoid content in the colon. Eighteen out of twenty-five *C. perfringens* strains, examined for their toxin genotypes, proved to be toxin type A, while 7 out of 25 strains showed the α and β2 toxin genes in combination. The rotavirus antigen and *Eimeria* oocysts were detected from healthy rabbits (specimens of the day after weaning and one week later) in about 15% of specimens examined, but their presence in the sick animals was not significantly associated with any cluster.

Key Words: *Clostridium*, Cluster analysis, Enteropathy, *Escherichia coli*, rabbit.

INTRODUCTION

Enteric disorders are the main factor responsible for economic losses in rabbit production in European countries (Nouaille, 2006; Rosell, 2006; Grilli *et al*., 2006; Rosell *et al.,* 2009). The importance of the problem has not changed compared with the past. In the 1980s, Peeters (1987) quantified the loss due to digestive diseases in Belgian rabbitries at approximately 70%. Specific enteritis, with clear disruption of the intestinal cells, liquid intestinal content and diarrhoea, can be caused by infectious agents, such

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Received *July 2009* - Accepted *July 2010*

as pathogenic *Eimeria* or enteropathogenic *E. coli* (EPEC). The *eae* gene, encoding the intimin protein, and the *afr/2* gene, encoding a fimbrial adhesin, are the main genetic markers used to define rabbit EPEC strains (Milon *et al*., 1999). Another way to determine *E. coli* enteropathogenicity is bio-/serotyping, which led to establishing a link between bio-/serotype and high mortalities. In Italy, the presence of serotype O103 together with the rhamnose-negative fermentation character was also associated with pathogenicity in weaned rabbit *E. coli* strains (Agnoletti *et al*., 2006). This serotype is also present in France and Spain, while *E. coli* O15:H- and O109:K-:H2 serovars are prevalent, respectively in weaned and suckling rabbits, in Belgium and the Netherlands, and the O26:H11 type is found in weaned rabbits in France (Milon *et al*., 1999). In a number of cases, digestive disease may be promoted by feeding mistakes, environmental or social stresses and misuse of antimicrobials, triggering dysbacteriosis, involving low-enteropathogenic *E. coli*, and/or *Clostridium spiroforme* and other infectious agents such as rotavirus, or low-pathogenic *Eimeria* sp. In these cases, the digestive disorder may be considered multifactorial (Peeters, 1987). In the late 90s a new syndrome called ERE, Epizootic Rabbit Enteropathy (Enteropatìa Mucoide (Rosell, 2000); Enteropatia Enzootica (Alberti, 2006)) began to spread in European countries (Licois *et al*., 2005). Under experimental conditions the disease is characterised by the clinical signs of borgorygmus ("rumbling noise"), distension of the whole intestinal tract, including the stomach, with gas and fluid, sometimes associated with caecal paresis and the presence of mucus in the colon (Licois, 2004; Licois *et al*., 2005). Under field conditions, the feature most often reported is caecal impaction and the presence of mucoid substance in the colon (Rosell, 2000; Gallazzi, 1998). In any event, a suddenly high mortality rate at the average age of 6 to 8 wk (4 to 14 wk) is recorded. The absence of intestinal inflammation, especially at caecum level, is the main feature of the syndrome that differentiates it from the acute enteritis or enterotyphlitis observed during colibacillosis, coccidiosis and *C. spiroforme* iota-like enterotoxaemia. The role of *Clostridium perfringens* as aetiological agent of ERE has been investigated (Marlier *et al*., 2006), but no aetiological agents of ERE have been detected to date (Dewrée *et al*., 2007; Huybens *et al.*, 2009), although a bacterial (anaerobic, air-tolerant bacterium) or bacterial toxin origin has been suggested (Licois and Marlier, 2008).

This paper describes an observational study of spontaneous rabbit enteropathies in a rabbit production facility, carried out by using standardised binomial checklists of clinical signs and enteric lesions, followed by data processing with a cluster analysis. The objective of the cluster analysis was to identify homogeneous groups of animals according to their clinical and pathological variables and then to apply the common statistical association techniques between the animal groups (clusters) and laboratory outputs.

MATERIALS AND METHODS

Animals and feeding

In a problematic rabbitry, housing 2000 to 8000 weaned rabbits per cycle, located in Teramo province (Abruzzi region, Central Italy), for 15 cycles of fattening rabbit production, a 20 mo health surveillance aiming to study enteric pathologies was carried out by separation, within the fattening unit, of a group of rabbits which were reared with the same density as the batch of origin and the same feeding regime except for antimicrobial supplementation (non-medicated groups). Briefly, per cycle, up to a hundred (see Table 2) 33 to 37 d old New Zealand or Californian commercial hybrids, weaned the same day, were housed in a separate row of cages, 2 to 3 animals per 30×40 cm cage. Animals were fed *ad libitum* with fodder containing 16% crude protein, 4.8% crude fat and 17% crude fibre, and only supplemented with Robenidine (66 ppm). After the 55-60 d of age, the animals were fed with fodder containing 15.8% crude protein, 5.2% crude fat and 16.50% crude fibre. In the source batch, the mortality rate due to digestive causes and the average daily temperatures were recorded for each production cycle. In the non-medicated

groups, the enteric morbidity rate, feed intake and occurrence of bloating, rumbling noise and diarrhoea were recorded. The day after weaning (time T0) and a week later (time T1), 2 to 4 animals from the separated group were randomly selected, clinically examined, euthanised, and subjected to a pre-defined necropsy protocol. One or two days after the first enteric symptom (time T2) and 4-5 d after the start of the enteric symptoms (time T3), all, or if more than 10 animals were showing disease symptoms, up to 10 diseased live animals were selected and subjected to the protocols described above. Moreover, 1 or 2 clinically healthy rabbits were randomly selected and subjected to the same protocols by way of internal negative controls for T2 and T3. The protocols were carried out in compliance with Directive 86/609/EEC on the welfare of animals used for experimental purposes. The untested animals from the separate group were reared and marketed together with the batch of origin.

Clinical and necropsy examinations

The animals subjected to clinical and necropsy protocols were examined by using two checklists with binomial responses (yes/no answer to a list of 54 variables, symptoms or lesions). The clinical form consisted of the following sections (and variables): animal identification, clinical examination of the digestive system (diarrhoea, abdominal bloating, rumbling noise, caecal paresis, presence of mucus in the faeces) and characteristics of the diarrhoea (watery, haemorrhagic, mucoid). The necropsy form consisted of specific sections (and variables) regarding lesions of stomach, small intestine, caecum and colon (distension, liquid/gas/mucoid content, lack of content, impaction, inflammation, and necrosis). The presence of a symptom or lesion was scored with the attribute mild, medium or severe.

Microbiology

Bacteriological examination of intestinal content. Approximately 1 mL of small intestine and 1 mL of caecal contents of all necropsied animals were each diluted in 2 mL of meat broth and subjected to aerobic and anaerobic microbiological analysis. Briefly, for aerobic analysis a loopful of material taken from the diluted specimen was inoculated onto Blood agar (BioMérieux SA, Marcy l'Étoile, France), Gassner medium agar (Biolyfe SA, Milano Italy) and Mannitol Salt agar (Biolyfe SA) and incubated under aerobic conditions at 37±1°C for 24 to 72 h; for anaerobic analysis, about 1 mL of diluted specimen was inoculated into thioglycollate broth (BioMérieux SA,) and, after incubation at 37±1°C for 24 h, a loop taken from each tube was inoculated onto Blood agar and incubated under anaerobic conditions (AnaeroGen™ Oxoid Ltd, Hampshire, England) at 37±1°C for 24 h. The suspect colonies from the aerobic and anaerobic cultures were sub-cultured onto Blood agar, incubated at 37±1°C for 24 h respectively under aerobic or anaerobic conditions and verified with Gram stain, catalase, and oxidase tests. The isolates were identified by miniaturised commercial kits (API 20E™, API 20NE™, API 20A™, BioMerieux SA,). The *E. coli* strains were placed in Soy agar and stored at 4±2°C; the *C. perfringens* strains were suspended in beads and stored at −80±2°C for the subsequent tests.

Clostridium spiroforme detection. Approximately 1 mL of the caecal content of each necropsied animal was tested by bacterioscopy to detect *C. spiroforme*, as described by Holmes *et al*. (1988). Positive specimens were classified as presence of "rare elements" or "several elements", on the basis of the detection, within a horizontal line of fields, respectively, of ≤1 bacterial element per microscopic field and >1 element in all microscopic fields.

E. coli serotype and biotype detection. The *E. coli* isolates were serotyped according to the recommendations of Blanco *et al*. (1996), using a set of 20 monospecific antisera targeting the following *E. coli* somatic antigens: O2, O8, O10, O15, O20, O22, O26, O49, O75, O86, O92, O103, O109, O128, O132, O139, O141, O149, O154 O153 and O157. The *E. coli* biotype was identified on the basis of sorbose, dulcitol,

raffinose, sucrose and rhamnose fermentation, according to the method described by Camguilhem and Milon (1989).

E. coli eae and *afr/2* gene detection. A sample from the collection of *E. coli* isolates (69 out of 138 strains collected) was tested for the presence of *eae* and *afr/2* genes by a polymerase chain reaction protocol, as described by China *et al*. (1996) and Penteado *et al*. (2002).

Clostridium perfringens toxin gene detection. A sample from the collection of *C. perfringens* isolates (25 out of 64 strains collected) was tested for *cpa*, *cpb1*, *cpb2*, *cpe*, *cpi* and *cpetx* genes encoding for α, β1, β2, enterotoxin, i and e toxins respectively, with polymerase chain reaction as described by Baums *et al*. (2004) and Yoo *et al*. (1997), and as performed by Cocchi *et al*. (2007).

Eimeria oocysts detection. A mucosal smear taken from the small intestine and caecum of each selected animal was examined on a slide with a 24×24 mm coverslip by optical microscope to detect *Eimeria* oocysts. Positive specimens were semi-quantitatively classified as showing "few oocysts" (countable elements not in all fields) or "several oocysts" (countable or uncountable elements in all microscopic fields).

Virological examination. Approximately 50 mL of intestinal content was taken from the small intestine, caecum and colon of each selected animal, pooled per animal, diluted 1:3 in PBS (if dry), filtered, and stored at −20±2°C. A sample of 134 out of 263 pooled intestinal contents was examined to detect rotavirus antigen with an ELISA test (Cypress Diagnostics®, Belgium), used in similar studies by Marlier *et al.* (2006) and Szalo *et al*. (2007).

Data processing and statistics

To enable the identification of homogeneous groups, the clinical and pathological variables were first reduced to six macro variables or profiles (A to F) by setting, for each segment of the digestive system, the determinants of a pathological condition, according to the criteria reported in Table 1.

A Two-Step Cluster Analysis (Anderberg, 1973; SPSS, 2006) was then applied to the six profiles in order to group the animals in homogeneous clusters. The analysis used the log-likelihood measure to calculate the distance between clusters and the Akaike Information Criterion (AIC) as grouping criterion. The positive or negative contribution of each profile to cluster making was tested by the chi-square test with Bonferroni correction. Finally, the chi-square test for independence was applied to assess the statistical significance of the associations between clusters and laboratory outcomes. The chi-square test was also

Table 1: Criteria used in determining clinical and pathological profiles

	A	B	C	D	E	F
Perineal smearing	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No
Bloating/rumbling noises	Yes	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No
Stomach, liquid content	Yes	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No
Small intestine, distension/liquid content	Yes/No	Yes	Yes/No	Yes/No	Yes/No	Yes/No
Small intestine, inflammation	No	Yes/No	Yes	Yes/No	Yes/No	Yes/No
Caecum, liquid content	Yes/No	Yes/No	Yes/No	Yes	No	Yes/No
Caecal partial/total impaction	Yes/No	Yes/No	Yes/No	No	Yes	Yes/No
Caecum, inflammation	No	Yes/No	Yes	Yes/No	Yes/No	Yes/No
Colon, mucoid content	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	Yes

The variables not listed in the table were to be answered as yes/no for each profile.

used to assess the associations between serotype, biotype, rhamnose fermentation character and *eae* gene presence in *E. coli* isolates.

RESULTS

Animals, batches examined and clinical examinations

Table 2 reports the main general data of the study regarding the batch of origin, separated group, number of animals examined, daily mean temperature, age average of starting and end of enteric symptoms per cycle. During 15 cycles, 1217 rabbits were subjected to health monitoring and 263 to the diagnostic protocols, 91 of them at time T0 and time T1 (no enteric symptoms), 154 at time T2 and time T3 (early and later cases of enteric symptoms) and 18 as internal negative controls of T2 and T3. The total mortality of the batches of origin showed a reverse trend to the mean daily temperatures, while the total morbidity of the non-medicated groups showed a trend independent of the daily temperature (Figure 1). On average, animals in the non-medicated groups started to exhibit enteric symptoms at the age of 49 d (46 to 59 d) and the last enteric symptoms were recorded at the age of 58 d (50 to 72 d). For eight cycles, the duration of the enteric disease was within the standard deviation from the general average of 13.5 d, but deviated from the general average by more than the standard deviation in the other cycles. Abdominal bloating, rumbling noise, weight loss and perineal smearing were the main constant signs at the start of the enteric disease.

Cluster analysis of clinical and enteropathological profiles

Table 3 shows the positive (YES) or negative (NO) percentage contribution of pathological profiles, and the corresponding chi-square value, to construct the four clusters obtained by cluster analysis. The profiles C and D contributed positively to Cluster 1 while A, B and E contributed negatively. Cluster 1 groups, with statistical significance, diseased animals with enteric inflammation, liquid content in the caecum and without bloating, caecal impaction or liquid content in the small intestine. Cluster 2 groups diseased animals with bloating/liquid content in the stomach (profile A) and liquid content in the caecum (profile D), without enteric inflammation (profile C) or caecal impaction (profile E). Cluster 3 groups animals to which all profiles contributed negatively. Cluster 4 groups diseased animals with dilation/liquid content of the small intestine (profile B), caecal impaction (profile E), mucus content in the colon (profile F) and without liquid content in the caecum (profile D).

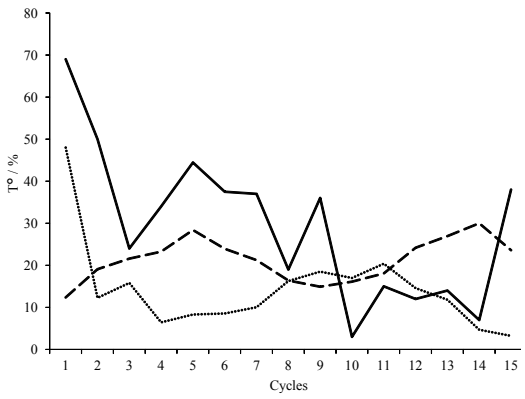

Figure 1: Daily mean temperatures (°C) (- -), total mortality (%) of batches of origin (·····) and total morbidity (%) of the non-medicated groups during the observation period (——).

Microbiology

Aerobic and anaerobic microbiological detection. One hundred and thirty-eight *E. coli* and 8 other aerobic bacteria (3 *Klebsiella sp.*; 2 *Bacillus sp.*; 1 *Pseudomonas*; 2 *Bacillus sp.*/*Acinetobacter sp.*, *Bacillus sp.*/*Klebsiella sp.* mixed isolates), from 104 animals, and 106 anaerobic bacteria (comprising 64 *Clostridium perfringens*; 29 *Clostridium sp.*; 9 *Clostridium butyricum*; 1 *Clostridium sordellii*; 1 *Clostridium ramosum* and 2 unidentified anaerobic bacteria), from 87 animals, were isolated from the group of diseased animals (specimens T2 and T3). *E. coli* and/or

Table 2: General data of the study.

	Source batch				Separated group			Examined animals					Enteric symptoms		
			Environmental data						Sampling time (No)[1]				Age average (d)		
Cycle	No	Mortality (%)	Period (mo)	Daily mean temperature	No.	Weaning age (d)	Diseased No (%)	No	T0	T1	T2 (k)[2]	T3 (k)	Starting	End	Duration (d)
1	2.510	48	Feb-Mar	12.4	100	37	69 (69.0)	24	4	4	8 (1)	8 (1)	46	71	25
2	3.532	12.3	Mar-May	19.1	58	34	29 (50.0)	17	4	2	9 (1)	2 (1)	46	66	20
3	2.010	15.8	Apr-Jun	21.6	50	36	12 (24.0)	12	2	1	1	8 (1)	46	50	6
4	3.200	6.4	May-Jun	23.3	50	35	17 (34.0)	16	1	1	4	10 (1)	48	56	13
5	3.660	8.3	Jun-Jul	28.4	27	35	12 (44.4)	13	1	1	6	5 (1)	51	56	14
6	3.255	8.5	Aug-Sep	23.9	32	35	12 (37.5)	10	0	4	1	5 (1)	47	56	16
7	3.060	10	Sep-Oct	21.2	100	33	37 (37.0)	20	2	4	8	6 (1)	48	58	16
8	2.280	16.3	Oct-Dec	16.4	100	36	19 (19.0)	23	4	4	9 (1)	6	47	54	13
9	2.080	18.5	Dec-Feb	14.9	100	37	36 (36.0)	30	4	4	8 (1)	14 (1)	58	72	24
10	824	17	Jan-Mar	16.1	100	36	3 (3.0)	11	4	4	0	3	54	59	7
11	2.580	20.4	Feb-Apr	18.1	100	36	15 (15.0)	15	4	2	5 (1)	4	46	52	12
12	3.844	14.6	Apr-May	24.2	100	35	12 (12.0)	20	4	4	5	7(1)	59	63	7
13	1.760	11.8	May-Jul	26.9	100	35	14 (14.0)	19	4	4	5 (1)	6 (1)	50	61	12
14	7.389	4.7	Jul-Sep	30	100	36	7 (7.0)	9	4	2	1	2	52	55	7
15	4.046	3.2	Sep-Oct	23.6	100	36	38 (38.0)	24	4	4	8 (1)	8 (1)	51	58	10
Total	46.030				1.217	35.5[3]	332	263	46	45	78 (7)	94 (11)	49[4]	58[4]	13.5[3]

[1] Sampling time: T0, day after weaning; T1, a week after weaning; T2, 1-2 d after the first enteric symptom; T3, 4-5 d after the start of the enteric symptoms. [2](k): number of internal negative control, [3] average, [4] median.

Table 3: Positive (YES) and negative (NO) percentage contribution of clinical and pathological profiles of diseased animals in the determination of clusters.

	Cluster 1 (No=39)		Cluster 2 (No=56)		Cluster 3 (No=29)		Cluster 4 (No=48)	
Profile	Yes/No	*P*	Yes/No)	*P*	Yes/No	*P*	Yes/No	*P*
A. Bloating/rumbling noises, stomach, liquid content; absence of inflammation	NO (41.9)	<0.001	YES (54.8)	<0.001	NO (33.7)	<0.001	YES (42.9)	0.18. NS
B. Small intestine, distension/liquid content	NO (35.1)	0.002	YES (43.7)	0.07 NS	NO (26.8)	0.02	YES (45.0)	0.006
C. Small intestine/caecum, inflammation	YES (72.0)	<0.001	NO (41.4)	<0.005	NO (22.7)	0.02	YES (28.0)	0.99 NS
D. Caecum, liquid content	YES (44.8)	<0.001	YES (55.2)	<0.001	NO (34.1)	<0.001	NO (56.5)	<0.001
E. Caecal partial/total impaction	NO (29.7)	0.001	NO (43.2)	<0.001	NO (26.1)	0.001	YES (100)	<0.001
F. Colon, mucus content	NO (26.6)	0.26 NS	NO (33.6)	0.92 NS	NO (22.7)	0.02	YES (58.3)	<0.001

No: number of diseased animals, *P*: Chi-square value of significance, NS: Not significant.

Clostridium genus strains were always isolated in all cycles. More particularly, *C. perfringens* and *E. coli* were simultaneously present in 13 to 15 cycles.

E. coli serotype and biotype detection. Table 4 shows the distribution of *E. coli* serotypes and biotypes. The main serotypes were NT (non-typable *E. coli* strains, 48.6%), O103 (16.7%), O49 (15.2%), O2 (8.0%) and O141 (6.5%). The main biotypes were B30 (39.9%), B31 (14.5%), B12 (13.0%), B19 (9.4%) and B28 (8.0%). On the basis of the character of rhamnose fermentation, 15.2% of biotypes proved rhamnose negative and 84.8% rhamnose positive. A statistically significant association was found between serotype O103 and the rhamnose-negative character ($P<0.001$) and between NT strains and rhamnose-positive

Table 4: *E. coli* serotype and biotype distribution in 138 strains isolated from diseased animals.

	E. coli biotype																
	B4	B10	B12	B14	B17	B18	B19	B20	B21	B22	B25	B27	B28	B29	B30	B31	Total
E. coli serotype																	
NT	1	1	2				10	2		1	1	1	5	6	28	9	67
O103			14	1			1						6			1	23
O141										2					6	1	9
O157															1		1
O2					1	1	2		1					1	1	4	11
O22			1														1
O49			1												15	5	21
O75															1		1
O86						1									3		4
Total	1	1	18	1	1	2	13	2	1	3	1	1	11	7	55	20	138

NT: Non-typable

Table 5: Presence of microbiological agents, Rotavirus antigen and *Eimeria* oocysts in the intestinal content of healthy animals.

Specimen	No	Aerobic	%	Anaerobic	%	*Eimeria*	%	Rotavirus[1]	%
T0 (on 5th wk)	46	1	2.2	11	23.9	1	2.2	0	0.0
T1 (on 6th wk)	45	3	6.7	5	11.1	13	28.9	4	33.3
Total	91	4	4.4	16	17.6	14	15.4	4	15.4

[1]Rotavirus antigen test was carried out on a sample of 26 intestinal contents (14 on T0, 12 on T1)

character. An association between serotype O49 and rhamnose-positive character was also found, but it was not statistically significant.

E. coli eae and *afr/2* gene detection. A sample of 69 *E. coli* isolates (50% of *E. coli* strains collection), was selected for *eae* and *afr/2* gene detection. Twenty eight strains came out positive for both *eae* and *afr/2* genes. A statistically significant association was found between *eae* gene and serotype O103 variables (P=0.002) and between *eae* gene and rhamnose-negative character (P=0.02). No statistically significant associations were found between the NT type (the most representative among *E. coli* serotypes) and *eae* gene detection.

Clostridium spiroforme detection. *C. spiroforme* was detected in 71 diseased rabbits (41.3% of total specimens T2 and T3), 25 of them with detection of "several elements" and 46 with "rare elements".

Clostridium perfringens toxin genotyping. A study of toxin genes was carried out on a sample of 25 *C. perfringens* isolates (39.1% of strains isolated). Only *cpa* and *cpb2* toxin genes were found. Specifically, the *cpa* gene was found on its own in 18 strains, whereas *cpa* in combination with the *cpb2* gene was found in 7 strains.

Eimeria oocysts detection. *Eimeria* oocysts were detected in 90 diseased rabbits (52.3% of total T2 and T3 specimens), 73 of them with detection of "few oocysts" and 17 with "several oocysts".

Virology. Rotavirus antigen test was carried out on 134 intestinal contents (50.1% of all selected healthy and diseased animals). Overall it was found in 23 samples (17.2%). No obvious difference was found in the distribution of positive results between specimens from healthy (T0, T1) and diseased animals (T2, T3).

Early presence of agents in the examined animals. Table 5 contains data concerning the presence of aerobic and anaerobic bacteria and *Eimeria* oocysts in the small intestine and caecum contents of all examined animals at time T0 and T1 (absence of enteric disease). This table also shows the results for the presence of rotavirus antigen in 26 samples examined out of 91 intestinal contents at the same sampling times. The aerobic bacteria proved to belong to the genus *Klebsiella* (1), *Enterobacter* (1) and *Bacillus* (2), and the anaerobic bacteria to the genus *Clostridium* (*C. spiroforme*, 1; *C. ramnosum*, 1; *Clostridium bifermentans*, 1; *C. butyricum*, 2; *Clostridium sp*, 11). The rotavirus antigen was detected starting from time T1 (4 positive out of 12 specimens at 6 wk old animals).

Statistical analysis of laboratory results

Table 6 shows the observed frequencies of the main laboratory outcomes and their association with the clusters of pathological profiles of diseased animals. For each cluster, frequencies significantly higher or lower than the expected ones are indicated with the corresponding chi-square value. The *E. coli* positive, *E. coli* O103 type positive detection and the finding of 'several' *C. spiroforme* were significantly associated with cluster 1 (to which enteric inflammation and liquid content in the caecum variables contributed). The finding of 'rare' *C. spiroforme* was significantly associated with cluster 2 (to which bloating/liquid content

Table 6: Frequencies of laboratory outcomes, their distribution within the 4 clusters of diseased animals and significance of the association between laboratory outcome and membership of the cluster (*P*-value).

	Cluster					
Laboratory outcome	1	2	3	4	Total	*P*-value
E. coli positive	33(+)	35	7(−)	29	104	<0.001
E. coli negative	6(−)	15	20(+)	14	55	<0.001
Other aerobic isolate	0	6	2	5	13	NS
E. coli O103 serotype positive	10(+)	6	2	2	20	0.03
E. coli O103 serotype negative	21(−)	29	6	28	84	0.03
E. coli eae+afr/2 genotype positive	9	10	2	7	28	NS
E. coli eae+afr/2 genotype negative	13	14	3	11	41	NS
C. perfringens positive	7	16	3	20(+)	46	0.01
Clostridium sp. positive	12	14	4	11	41	Ns
Negative for anaerobes	20	26	22(+)	17	85	0.01
C. perfringens cpa genotype	2	7	1	8	18	NS
C. perfringens cpa + *cpb2* genotype	1	3	0	3	7	NS
Rare *C. spiroforme*	12	21(+)	1(−)	12	46	<0.001
Several *C. spiroforme*	10(+)	7	0(−)	8	25	<0.001
C. spiroforme negative	17(−)	28	28(+)	28	101	<0.001
Few oocysts of *Eimeria*	14	30	5(−)	24	73	0.05
Several oocysts of *Eimeria*	4	4	5	4	17	NS
Eimeria oocysts negative	21	22	19	20	82	NS
Rotavirus positive	7	6	0	6	19	NS
Rotavirus negative	17	33	11	28	89	NS

(+) frequency observed greater than expected and chi-square test significant at threshold level of $\alpha \leq 0.05$.
(−) frequency observed less than expected and chi-square test significant at threshold level $\alpha \leq 0{,}05$.
NS: Not significant: observed chi-square values below critical value.

in the stomach and liquid content in the caecum variables contributed). The absence of *E. coli*, anaerobic bacteria and *C. spiroforme* was significantly associated with cluster 3 (which groups negative controls and diseased animals with minor lesions). The isolation of *C. perfringens* was significantly related to cluster 4 (to which liquid content/dilation of small intestine, caecal impaction and mucus content in the colon variables contributed). The observed value was not significant when the chi-square test was applied to the frequencies of the aerobic isolates other than *E. coli*, *E. coli eae* and *afr/2*-genotype, *Clostridium sp.*, *C. perfringens cpa* or *cpa+cpb2*-genotype, rotavirus antigen and *Eimeria* oocysts outcomes. The chi-square test was also performed to establish whether there was an association between the mean age at the start of enteric disease, the time of sampling (T2, T3) and the clusters. This association was not significant either.

DISCUSSION AND CONCLUSION

The experimental model used in this study proved suitable for its purposes. Separate rearing of a group of animals, with the same procedures as the source batch except for medication of the feed, promoted spontaneous exacerbation of the enteric disorders. From the standpoint of representativity of the obtained results, nearly 50% of the diseased animals were examined during the 20 mo observation period, which

included all months of the year. As shown in Figure 1, the mortality values in the batch of origin form an inverse curve to the mean daily temperatures in the shed. The reduction of feed intake caused by the increase of mean temperature probably induced a self-rationing of rabbit feeding. After weaning, the feeding reduction was considered an effective tool for managing enteric disorders, especially during experimental ERE (Boisot *et al*., 2003). On the contrary, in the non-medicated groups the morbidity curve is independent of temperature, indicating that in those groups, in absence of an antimicrobial supplementation, the increase of pathogen pressure is the factor which probably contributes most to morbidity and disease spread. On average, the animals began to present an enteric disorder at the age of 7 wk (46 to 59 d). This age is compatible with Enzootic Rabbit Enteropathy (Licois *et al.*, 2005), but was not significantly associated with any cluster of clinical pathological profiles, indicating that this is rather the age of greatest susceptibility of rabbits to spontaneous enteropathies. The mean age of starting and the duration of the enteropathic event are apparently not related to the months of the year. Neither is the morbidity rate, indicating that the spread of enteropathies within the non-medicated group is apparently also related to the susceptibility of the rabbit, as the digestive apparatus reaches full functional and immunological maturity around the 8th wk (Fortun-Lamonthe and Boullier, 2007). The period that elapses between weaning, at 35 d, and the start of the enteropathic event, at 49 d, is the time when pathogens, under stress for diet change, may multiply in the gut. In the specimens from selected animals at T0 and T1 (at 35 to 42 d), an aerobic, non-coliform flora is already found in 4.4% while Clostridia other than *C. perfringens* and *Eimeria* oocysts are found, respectively, in 17.6% and 15,4% of the examined animals. At this stage, rotavirus antigens were also detected in 4 out of 26 examined rabbits.The early symptoms of enteropathy found in the non-medicated groups, namely bloating/rumbling noises, faecal smearing of the perineum and weight loss, are compatible with Enzootic Rabbit Enteropathy (Licois *et al.*, 2005). The spontaneous development of enteric disorders led to more defined profiles of inflammatory enteric disease (enterotyphlitis), non-inflammatory enteropathy, caecal impaction and mucoid enteropathy, which were often found combined.

Cluster analysis enabled us to group the clinical-pathological profiles of the animals suffering from enteric syndromes into three groups or clusters, which comprised about 83% of cases (cluster 3, to which all pathological variables contributed negatively, is excluded). No cluster prevailed over another in the various cycles examined. The animals showing inflammation of the small intestine and caecum and those that had liquid content in the caecum significantly contributed to the first cluster. This cluster was significantly associated with *E. coli*, *E. coli* O103 positive detection in the contents of the caecum and/or the small intestine and the presence of *C. spiroforme* in the caecum ("several elements" variable). Serotype O103, which was prevalent among the typable strains, was significantly correlated with the negative rhamnose-fermenting character and the presence of *eae* gene, thus being confirmed as an indicator of pathogenicity (Agnoletti *et al*., 2006). In our study, the rhamnose-negative B12 biotype was significantly associated with O103 serotype (14 out of 23 strains). In Italy, the O103-B12 *E. coli* strain is one of the main agents responsible for colibacillosis outbreaks (Agnoletti *et al*., 2004).

The *C. spiroforme*, 'several elements' variable, was also significantly associated with cluster 1 (10 cases out of 39 animals). Although it was considered responsible for specific enteritis, in our study its detection should be considered as an overlapping agent in the cases of enterotyphlitis, confirming the observations of various authors (Licois and Marlier, 2008; Agnoletti *et al*., 2006).

To the other two clusters, named 2 and 4, which grouped about 60% of diseased animals, respectively, the rabbits with bloating/rumbling/liquid content in the stomach, absence of intestinal inflammation and/or liquid content in the caecum (cluster 2) and rabbits with liquid content in the small intestine and/or caecal impaction and/or mucoid content in the colon (cluster 4) contributed significantly. The variables which contributed to define cluster 2 and 4 are compatible with ERE (Licois *et al.*, 2005). From a diagnostic

standpoint, cluster 2 is significantly associated with the presence of *C. spiroforme* ("rare elements" variable), while cluster 4 is significantly associated with the presence of *C. perfringens*. The cases positive for *C. perfringens* belonging to cluster 2 were numerically high, though not on a significant level.

The unclassified toxin type containing both *cpa* and *cpb2* genes is also considered by other authors to be associated with ERE, especially with caecal impaction (Le Normand *et al*., 2003; Perez de Rozas *et al*., 2005). In our study, the data did not support this association.

The hypothesis of *C. perfringens* as aetiological agent of ERE, according to Koch's postulate, could not be corroborated by earlier studies (Marlier *et al*., 2006) although it was acknowledged that this agent might play a role in high mortalities observed during spontaneous ERE (Licois and Marlier, 2008).

To conclude, the cluster analysis proved to be a powerful tool for the study of rabbit enteric pathologies typically characterised by clinical symptoms and pathological lesions not specific for a definite syndrome. Particularly, the cluster analysis enabled us to manage a higher number of variables for a large number of animals. The application of cluster analysis in rabbit pathology might contribute to identifying risk factors and aetiological agents in epidemiological studies carried out on a large scale.

Acknowledgements: This study was supported by a financial contribution from Avitalia, Unione Nazionale Associazioni di Produttori Avicunicoli, Forlì, Italy, as part of the programme entitled "Miglioramento della qualità, della gestione dell'offerta delle produzioni cunicole e di rafforzamento dei rapporti di filiera. Azione 4.3". Our thanks go to breeder Leta Covelli and Dasco srl for supplying the rabbits, to our colleague Romolo Salini and to Fabrizio Agnoletti of the Istituto Zooprofilattico Sperimentale del Veneto, Trevise, Italy.

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