TOXIN-GENOTYPING OF *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM RABBITS WITH ENTERIC DISEASE

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ABSTRACT

In commercial rabbitries, enteritis are considered a major cause of economic loss. Among the infectious agents responsible for enteritis/enterotoxaemia, *Clostridium. perfringens, C. difficile* and *C. spiroforme* are the most common isolates even if the role played by *C. perfringens* in rabbit enteritis is still under debate. In one commonly-used toxin typing scheme, *C. perfringens* isolates are assigned to one of five types (A-E), depending on the production of the following major toxins: α, β, ε, and ι. Other toxins, such as enterotoxin, traditionally associated with type A, and toxin β2, are involved in the pathogenesis of enterotoxaemia in animals. Type A *C. perfringens* strains, for example, are commonly found in the intestinal tract of both humans and animals. In the past, type E *C. perfringens* has been considered the etiological agent of rabbit enteritis, even if various authors failed to isolate it from the caecum of diseased rabbits. The pathogenic role of *C. spiroforme,* mediated by the production of an iota-like toxin, was demonstrated. This binary toxin has a functional and antigenic resemblance with the binary iota toxin, typical of type E *C. perfringens*. Enteritis caused by clostridia are conditioned pathologies. Errors in diet, stress, and drug administration, in fact, can all cause alteration of bowel microflora that leads to an overgrowth of *Clostridium spp*. The aim of this study was to investigate the distribution of toxin types in *C. perfringens* field strains, isolated in rabbit enteritis. One hundred and fifty colonies of *C. perfringens*, each randomly chosen from a culture obtained by streaking the caecal content of diseased rabbits, were initially subjected to PCR for toxin type identification. In order to verify whether one or more *C. perfringens* toxin types was present in the rabbit gut at the same time, 14 animals positive for *C. perfringens* were studied and 5 different *C. perfringens* strains collected from each one were toxin-genotyped for a total of 70 strains. The results showed type A to be the type most commonly recovered and the analysis of the distribution of the toxin types recovered from a culture confirmed the uniform distribution of toxin types; then one colony seemed to be representative of all the types recovered in the culture. This might prove useful in clostridiosis diagnostic procedure. A high percentage of the new toxin type A+β2 was observed to be present among toxin type A; the gene codifying for β2 toxin is revealed to be present in association with type E, too. The new toxin type E+β2 was therefore discovered in diseased rabbits. Moreover, the absence of enterotoxin poses no little amount of concern to public health, because this toxin is involved in human foodborne disease. Further studies are required to verify the exact role played in the pathogenesis of this conditioned pathology by both toxin type A and the newly discovered toxin, β2.

Key words: *Clostridium perfringens*, Toxin genotyping, Rabbit, Enteric disease.

INTRODUCTION

Enteritis are the major cause of disease in commercial rabbit industry. Diet, stress and management factors are also acknowledged to affect the incidence and spread of enteric disease. Infectious agents known to play a role include parasites, enteropathogenic *E. coli* (EPEC), *K. pneumoniae*, and *Clostridium spp* (Percy *et al*., 1993). Among the Clostridium spp. *C. spiroforme*, *C. difficile* and *C. perfringens* are the most commonly isolated.

C. perfringens, a Gram positive, sporulating anaerobic bacterium, is widespread in environmental matrices (soil, water) and is commonly found in the intestines of humans and animals. It does not present adherence or invasive properties in healthy intestinal mucosa (Petit *et al*., 1999). Alterations in the physiological equilibrium of the resident microflora (due to antibiotic therapy or improper feed) allow the colonization of toxigenic clostridia. This bacterial species is often involved in enteric disease, enteritis and enterotoxaemia in both humans and animals. *C. perfringens* can be sub-typed into five toxin types (A-E) on the basis of the production of the four major toxins: alpha, beta, epsilon and iota as reported in the following table (Songer, 1996).

Table 1: *C. perfringens* toxintypes and disease produced

C. perfringens type	Gene	Disease caused
A	α	Necrotic enteritis in poultry, clostridial myonecrosis, food poisoning
B	α, β, ε	Hemorrhagic enterotoxemia in adult sheep, lamb dysentery
C	α, β	Enterotoxemia in sheep, necrotic enteritis in neonatal pigs, goats, lambs, calves
D	α, ε	Enterotoxemia in lambs and calves
E	α, ι	Enterotoxemia in calves

Alpha (α) toxin is the principal lethal toxin of *C. perfringens*, and the gene *cpa* is chromosomally localized. Beta (β) toxin induces inflammation and necrosis of the intestinal mucosa, and its gene resides on a plasmid element. Toxin ε is necrotizing and lethal, and the ε toxin gene (*etx*) resides on an extrachromosomal element. Toxin ι is a binary toxin and acts intracellulary, modifying the actin cytosckeleton. Two new toxins have recently been described: toxin β2 and enterotoxin.

Enterotoxin is linked to human (foodborne and nonfood-borne gastrointestinal diseases) and animal diarrhoeas (Songer, 1996; Smedley *et al*., 2004). Most *C. perfringens* isolates carrying the enterotoxin gene (*cpe*) classify as type A isolates. The *cpe* gene can be present on either the chromosome or on a large plasmid (Collie *et al*., 1998).

Discovered by Gibert *et al.* (1997), toxin β2 is encoded from the *cpb*2 gene, carried on a plasmid. The *cpb2* gene can be found in all *C. perfringens* toxin types (Bueschel *et al*., 2003).

In this study we analyzed field strains of *C. perfringens*, in order to investigate the presence and distribution of all the toxin types in *C. perfringes* cultures, obtained from rabbit with enteric disorders.

MATERIALS AND METHODS

Bacterial strains

The content of the caecum of 150 rabbits, affected from enteric disorders, was inoculated onto a dish of PAB (Perfringens agar Base, to which 5% V/V sheep erythrocytes was added) with a sterile loop. At the end of incubation time in anaerobic chamber (MiniMACS Anaerobic Workstation, PBI), one suspected colony of *C. perfringens* from each PAB was identified by biochemical panel (API ANA; Biomerieux). Subsequently we chosen other 14 diseaded rabbits and the caecal content from each was inoculated onto a dish of PAB, as desribed above. In this case, at the end of the incubation time, 5 suspected colonies of *C. perfringens* from each plate were identified. Then both the first 150 colonies and the latter 70 colonies obtained, identified as *C. perfringens*, were submitted to molecular analysis in order to toxin genotyping. A total of 220 strains was considered.

Molecular analysis

Nucleic acid was extracted using the "Gene Elute Bacterial genomic DNA kit" (Sigma-Aldrich) commercial kit according to manufacturer's instructions and the DNA was subjected to a multiplex PCR to identify α, β, ε and ι toxins and to duplex PCR to determine β2 toxin and enterotoxin (Baums

et al., 2004; Yoo *et al*., 1997). The PCR primers and the lengths of the expected amplicons are listed in Table 2.

Table 2: Nucleotide sequence of the used primers, length of amplicon obtained

Toxin	Gene	Nucleotidic sequenze (5'-3')	bp
α	Cpa_for	AGT CTA CGC TTG GGA TGG AA	900 pb
	Cpa_rev	TTC CCT GGG TTG TCC ATT TC	
β	Cpb1_for	TCC TTT CTT GAG GGA GGA TAAT	611 pb
	Cpb1_rev	TGA ACC TCC TAT TTT GTA TCC CA	
ε	Cpetx_for	ACT GCA ACT ACT ACT CAT ACT GTG	541 pb
	Cpetx_rev	CTG GTG CCT TAA TAG AAA GAC TCC	
ι	Cpi_for	AAA CGC ATT AAA GCT CAC ACC	293 pb
	Cpi_rev	GTG CAT AAC CTG GAA TGG CT	
Enterotoxin (etx)	Cpe_for	GGG GAA CCC TCA GTA GTT TCA	506 pb
	Cpe_rev	ACC AGC TGG ATT TGA GTT TAA TG	
β2	Cpb2_for	CAA GCA ATT GGG GGA GTT TA	200 pb
	Cpb2_rev	GCA GAA TCA GGA TTT TGA CCA	

RESULTS AND DISCUSSION

The results of toxin genotyping of 150 strains of *C. perfringens* are listed in Table 3. Table 4 presents the toxin types distribution of the five colonies obtained from the same culture.

Table 3: Results of the toxin-genotyping of 150 *C. perfringens* field strains

Toxinotype	N	%	No. positive to *cpe* gene	%	No. positive to *cpb2* gene	%
A	149	99.33	0	0	37	24.8
B	0	0	0	0	0	0
C	0	0	0	0	0	0
D	0	0	0	0	0	0
E	1	0.66	0	0	0	0
TOT	150	100				

Table 4: Distribution of the toxin types of the five colonies recovered from the same PAB obtained streaking intestinal content of a single diseaded rabbit (number of positive colonies for the toxin type/total number of tested colonies)

	Rabbit	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Toxinotypes	A	0/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5	4/5	5/5	5/5
	B	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	C	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	D	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	E	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	E+β2	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	A+β2	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	1/5	0/5	0/5

These results permitted to define how toxin types are distributed in a diseased subject. The study showed an elevated presence of toxin type A, which appeared in 149 out of 150 strains typed (99.33%). Twenty-five percent of 150 strains contained the *cpb*2 gene yielding the toxin type A+β2. Distribution analysis revealed the type E to be associated with the *cpb2* gene, resulting in the newly described E+β2 toxin type. Furthermore, toxin types were observed to be distributed equally throughout the *C. perfringens*'s culture, and in only one case four colonies harbouring the *cpa* gene and one colony harbouring both the *cpa* and *cpb2* genes in the same animal was observed.

CONCLUSIONS

The toxin genotyping of 150 field strains has demonstrated the high prevalence of type A *C. perfringens*, whereas toxin type E appeared to have a marginal role in rabbits affected by enteropathy, and the same holds true for types B, C and D. Our results have also showed that the analysis of a

single colony chosen from a given culture seems to allow the characterization of all the *C. perfringens* toxin types in the caecal content. Only one toxin genotype in fact, was spread, even if the use of other techniques (e.g. PFGE) is required in order to establish the possible clonal origin. This uniformity might be explained by the transfer of extrachromosomal DNA between *C. perfringens* isolates (Brynestad *et al*., 2001). Each toxinotype of *C. perfringens* produces one or more toxins and can cause a specific syndrome. Therefore, the correct identification of *C. perfringens* toxin type is an important tool for epidemiological studies and for the development of effective preventive measures. The use of molecular techniques is suitable in order to identify the genes codifying toxins quickly and with high sensitivity and specificity, but in spite of this, is only able to establish the capability of a strain to produce toxins, so, the definitive diagnosis of *C. perfringens* enterotoxaemia should be confirmed by the detection of its toxins in the intestinal contents through the use of histological, enzymatic and proteomic techniques.

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