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DETECTION OF THE β2 TOXIN GENE FROM *CLOSTRIDIUM PERFRINGENS* ISOLATED IN DIARRHEIC PIGLETS

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ABSTRACT

According to the major toxin types that they produce, *Clostridium perfringens* isolates are currently classified as one of five biotypes. Enteritis associated with *C. perfringens* type A in pigs has been described in Europe and United States, but has not been reported in Brazil. A novel toxin called $\beta2^+$ has been recently identified and its encoding gene characterized. Preliminary studies suggested that strains producing β2-toxin are associated with necrotic enteritis in piglets, and with enterocolitis and typhlocolitis in horses. The clinical and microbiological features of four outbreaks of neonatal pig diarrhea in Brazil associated with *C. perfringens* type A, $\beta2^+$ are described in this report, as well as the genotype characterization of the isolated strains.

KEY WORDS: Enteritis, PCR, pig, *Clostridium perfringens*, bacteria, toxin.

RESUMO

DETECÇÃO DO GENE CODIFICADOR DE TOXINA β2 EM AMOSTRAS DE *CLOSTRIDIUM PERFRINGENS* ISOLADAS DE LEITÕES COM DIARREIA. Com base nas toxinas produzidas as amostras de *Clostridium perfringens* são classificadas em 5 biotipos. A enterite causada pelo *C. perfringens* tipo A em suínos tem sido descrita na Europa e nos Estados Unidos, porem não tem sido relatada no Brasil. Uma nova toxina denominada $\beta2^+$ foi recentemente identificada e seu gene codificador caracterizado. Estudos preliminares indicam que amostras de *C. perfringens* produtoras desta toxina estão associadas à enterite necrótica em leitões e a enterocolite em equinos. Os achados clínicos e microbiológicos de quatro surtos de diarréia neonatal em leitões associados a presença de *C. perfringens* tipo A, $\beta2^+$ são descritas neste artigo assim como a caracterização genotípica destes isolados.

PALAVRAS-CHAVE: Enterite, PCR, suíno, *Clostridium perfringens*, bactéria, toxina.

INTRODUCTION

Clostridium perfringens is the etiologic agent of multiple syndromes in domestic animals, some of the most important conditions that producers and veterinary practitioners have to face (SONGER et al., 1998). The various toxins produced by the bacteria play key roles in the pathogenesis of the disease and are divided into five biotypes, designated A through E, based on the production of alpha- (α-), beta (β-), epsilon- (ε-), and iota- (ι-) toxins. The α-toxin is produced by all types, β-toxin is produced by type B and C strains, ε-toxin is produced by type B and D strains, and ι-toxin is produced by type E strains (GARMORY et al., 2000). Different biotypes of *C. perfringens* are associated with different diseases. Type C, for example, is generally considered to be the primary cause of necrotic enteritis in piglets aged 0-2 weeks, while type A has been linked to enteric disease in suckling and feeding pigs with mild necrotic enterocolitis and villi atrophy (COLLINS et al., 1989; TAYLOR, 1999). In addition to the major toxins, other toxins may play a role in disease (GARMONY et al., 2000). A novel toxin produced by *C. perfringens*, named beta 2- (β2-) toxin, has recently been identified and its encoding gene characterized. This toxin is cytotoxic for intestinal cells and lethal for mice (GILBERT et al., 1997). Preliminary studies suggested that β2-toxin-producing strains are associated with necrotic enteritis in piglets and enterocolitis and typhlocolitis in horses (GILBERT et al., 1997; HERHOLTZ et al., 1999). Diarrhea associated

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with *C. perfringens* type A in pigs has been described in Europe and United States but has not been reported in Brazil (Collins et al., 1989; Taylor, 1999).

In this report we describe the clinical and microbiological features of four diarrheic outbreaks associated with *C. perfringens* type A in neonatal pigs in Brazil, as well as the genotype characterization of isolated strains.

MATERIALS AND METHODS

Animals

Between May and September 2000, four continuously farrowing to finish swine herds from south of Brazil experienced an outbreak of diarrhea in 1- to 5-day-old pigs. High morbidity and low mortality characterized the clinical illness, usually starting shortly after birth. Diarrhea was clinically non-responsive to treatment with gentamicin but responded well to penicillin therapy. The morbidity was not prevented or reduced by routine vaccination of sows against *C. perfringens* type C and B, and *Escherichia coli* pillus types K88, K99, 987P and F41. The intestines of 2 unmedicated, acutely diarrheic, 1- to 5-day old piglets from each herd were submitted to the Swine Pathology Laboratory of the School of Veterinary Medicine, University of São Paulo. The feces were foamy, fluid, yellow and showed in some cases a small amount of blood.

Bacteriological examination

Ileum sections were cultured on sheep blood agar and MacConkey's agar plates incubated for 24 hours at 37º C in an aerobic atmosphere, yielding pure growths of *E. coli*.

Anaerobic cultures of the ileum of each pig were obtained by inoculating pre-reduced sheep blood agar plates. These plates were incubated under anaerobic conditions for 48 hours at 37º C and then examined for the presence of colonies with the morphologic structure and hemolytic pattern of *C. perfringens*. All the anaerobic cultures yielded dense growths of *C. perfringens*, confirmed by the use of Gram stains, lecithinase and lipase reaction in egg-yolk agar and presence of storm clout in Litmus milk (Quin et al., 1994).

Virological and parasitological examination

Fecal samples were submitted to Rotavirus RNA detection by electrophoresis in polyacrylamide silver stained gel (PAGE), as described by Herring et al. (1982). Parasitological examination was conducted as described by Ogassawara et al. (1989).

DNA Extraction

Eight *E. coli* strains from each MaConkey plate were cultured in 2 µL of BHI broth for 24 hr and 200 mL of each bacterial suspension was submitted to DNA purification with the guanidium thiocyanate method described by Boom et al. (1990).

Five colonies of *C. perfringens* from each piglet were cultured in 10 mL of thioglycolate broth for 24 hr at 37º C. The DNA extraction was conducted with 200µL of bacterial culture treated with Lysozyme (140 µL of 100mg / mL) and Proteinase K (40µL of 20 mg / mL) for 1 hour at 37º C. The bacterial lysates were submitted to DNA purification with the guanidium thiocyanate method described by Boom et al. (1990).

E. coli DNA templates were tested by PCR for gene encoding enterotoxins (Sta, STb, LT), shiga-toxins (SLTI, SLTII and SLT2v), pillus K88, K99, 987P and F41, as described by Blanco et al. (1999).

The DNA templates of *C.perfringens* were submitted to multiplex and individual PCR assays for the detection of genes codifying α-toxin (cpa), β-toxin (cpb), ε-toxin (etx), ι-toxin (iA), enterotoxin (cpe) and β2-toxin (cpb2), as described in Table 1. Control DNA from reference strains *C. perfringens* type A (ATCC 3624), type C (ATCC 3628), type B (ATCC 3626) and type D (ATCC 3629) were included in every reaction.

RESULTS

All *E. coli* strains examined were negative for the genes codifying the virulence factor studied. None of 8 fecal samples were positive to Rotavirus or coccidia.

Forty *C. perfringens* strains isolated from 8 piglets were positive for cpa gene, confirming their identification as *C. perfringens* and all strains were positive for cpb2 gene (Type A $\beta 2^{+}$).

DISCUSSION

A diarrhea syndrome with clinical, epidemiological and microbiological characteristics as described in these cases has been previously reported in neonatal pigs in major swine-producing regions of the United States and Europe (Jestin et al., 1985; Collins et al., 1989). *C. perfringens* type A enterotoxicosis is a difficult diagnosis to substantiate, especially because the organism is part of the normal intestinal flora (Collins et al., 1989). Several findings in the present case, however, support this diagnosis, including the absence of other known enteropathogens, culture of dense growths of *C. perfringens* from the ileum of all pigs examined, characterization of isolated strains by PCR and the clinical responsiveness of pigs to antimicrobial therapy.

Table 1 - Specific oligonucleotide primers used for PCR amplification of five different *C. perfringens* toxin genes.

Gene	Primer	Sequence (5´-3´)	Anneal temperature	Fragment length	Reference
cpa	PL3	AAGTTACCTTTGCTGCATAATCCC	55º C	283bp	Fach and Popoff, 1997
	PL7	ATAGATACTCCATATCATCCTGCT			
cpb	B1	GCGAATATGCTGAATCATCTA	55º C	196bp	Meer et al, 1997
	B2	GCAGGAACATTAGTATATCTTC			
etx	1	GCGGTGATATCCATCTATTC	55º C	655bp	Meer et al, 1997
	2	CCACTTACTTGTCCTACTAAC			
iA	1	ACTACTCTCAGACAAGACAG	55º C	446bp	Meer et al, 1997
	2	CTTTCCTTCTATTACTATAGC			
cpe	1	GGAGATGGTTGGATATTAGG	55º C	233bp	Meer et al, 1997
	2	GGACCAGCAGTTGTAGATA			
cpb2	1	GAAAGGTAATGGAGAA	48º C	573bp	Herholtz et al, 1999
	2	GCAGAATCAGGATTTT			

The relationship between *C. perfringens* type A, $\beta2^+$, and piglet enteritis was described by GILBERT et al. (1997) and has been studied by several authors since then. KLAASEN et al. (1999) reported 44% (12/27) of isolates from diarrheic piglets as type A, $\beta2^+$ vs. 7% (2/27) of isolates characterized as type C, $\beta2^-$. GARMORY et al. (2000) described 82% (27/33) of *C. perfringens* isolates from pigs with diarrhea as positive to cpb2 gene and showed a significant association between type A, $\beta2^+$ strains and diarrhea in piglets.

Further investigations on the occurrence of cpb2-positive *C. perfringens* type A in Brazilian piglets with diarrhea and the toxic effects of β2 toxin in animal or in vitro models are necessary. In addition, the use of polymerase chain reaction (PCR) for genotype field isolates has provided a powerful tool to generate information to be used as basis for new prevention and control strategies.

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