

Detection of enterotoxin A and cytotoxin B, and isolation of *Clostridium difficile* in piglets in Minas Gerais, Brazil

Detecção da enterotoxina A e citotoxina B e isolamento de *Clostridium difficile* em leitões em Minas Gerais, Brasil

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ABSTRACT

Clostridium difficile has emerged as a major cause of neonatal colitis in piglets, displacing classic bacterial pathogens. However, there is no information regarding the distribution of this microorganism in pig farms in Brazil. In the present study, the presence of toxins A/B and of *C. difficile* strains in stool samples from 60 diarrheic or non-diarrheic newborn piglets (one to seven days old), from 15 different farms, was studied. The presence of toxins A/B was detected by ELISA and PCR was used to identify toxin A, toxin B and binary toxin gene in each isolated strain. *C. difficile* A/B toxins were detected in ten samples (16.7%). Of these, seven were from diarrheic and three were from non-diarrheic piglets. *C. difficile* was recovered from 12 out of 60 (20%) fecal samples. Of those, three strains were non-toxigenic (A⁻B⁻) and nine were toxigenic. Of the nine toxigenic strains, four were A⁺B⁺ strains and five were A⁻B⁺ strains. The presence of binary toxin observed in the present study was much higher (50%) than in previously reported studies. All three non-toxigenic strains were isolated from otherwise healthy piglets. The results suggest the occurrence of neonatal diarrhea by *C. difficile* in farms in Brazil.

Key words: Neonatal diarrhea, *Clostridium difficile*, enteritis, colitis.

RESUMO

Clostridium difficile tem sido relatado como o principal causador de colite neonatal em suínos. Apesar da crescente importância deste agente, não há dados sobre infecções causadas por *C. difficile* em suínos no Brasil. O objetivo do presente estudo foi detectar as toxinas A/B e isolar *C. difficile* a partir de 60 amostras de fezes de leitões diarréicos

ou aparentemente saudáveis, com no máximo sete dias de vida, e oriundos de 15 granjas diferentes. As toxinas A/B foram detectadas por ELISA e uma PCR multiplex foi utilizada para detecção dos genes responsáveis pela codificação das toxinas A, B e toxina binária. As toxinas A/B de *C. difficile* foram detectadas em dez amostras de fezes (16.7%). Dessas, sete eram de animais diarréicos e três de leitões aparentemente saudáveis. Foi possível isolar *C. difficile* em 12 das 60 (20%) amostras trabalhadas. Dessas, três estirpes eram não-toxigênicas (A⁻B⁻) e nove eram toxigênicas, sendo quatro caracterizadas como A⁺B⁺ e cinco como A⁻B⁺. O gene responsável pela codificação da toxina binária foi encontrado em 50% das estirpes isoladas, proporção superior ao relatado em estudos anteriores. Todas as estirpes não toxigênicas foram isoladas de animais não diarréicos. Os resultados encontrados sugerem a ocorrência de diarréia por *C. difficile* em granjas no Brasil.

Palavras-chave: diarréia neonatal, *Clostridium difficile*, colite, enterite.

INTRODUCTION

Clostridium difficile is a spore-forming, anaerobic, Gram-positive bacillus that has been recognized as an important bacterial pathogen in both humans and animals. According to BARTLETT (1992), *C. difficile* may be responsible for 95% of all pseudomembranous colitis cases and most cases of antibiotic-associated diarrhea in humans. Most isolates of *C. difficile* produce two types of toxins that damage the colonic epithelium: toxin A, an enterotoxin, and

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toxin B, a cytotoxin (VOTH & BALLARD, 2005). In addition to these two major toxins, it has been suggested that a binary toxin, also called *C. difficile* transferase (CDT), may be an additional important virulence factor (STUBBS et al., 2000). It consists of two independent unlinked proteins chains that are encoded by two separate genes, designated *cdtA* and *cdtB*, and according to SCHWAN et al. (2009), the binary toxin may increase the adherence and colonization of the bacterium.

In veterinary medicine, *C. difficile* has been implicated as a cause of enteric disease in a variety animal species including adult horses, foals, dogs and rabbits (BAVERUD, 2002). In swine, the importance of *C. difficile* as an agent involved in porcine neonatal diarrhea has increased, displacing classic bacterial pathogens (SONGER & UZAL, 2005). It has been suggested that *C. difficile* may be currently the most important uncontrolled cause of neonatal diarrhea in pigs (SCHWAN et al, 2009).

Laboratory diagnosis of *C. difficile* infection is based on detection of toxin A and/or toxin B by cell culture or by enzyme immunoassays (ELISA) (DELMÉE, 2001). Moreover, isolation and screening for toxin genes leads to a better understanding of transmission patterns and risk factors and is useful for detecting variant strains that produce only one of the major toxins. The evaluation of the distribution of these strains and the potential association with the occurrence of diarrhea are important factors for elucidating the epidemiology of this disease (ARROYO et al., 2007; BARBUT et al., 2005).

Despite of the importance of *C. difficile* as a swine pathogen, there are no current clear data concerning the distribution of this microorganism in pig farms in Brazil. Therefore, the aim of this study was to detect *C. difficile* A/B toxins and to isolate strains of *C. difficile* in stool samples from diarrheic and non-diarrheic piglets.

MATERIAL AND METHODS

Stool samples were collected from 60 piglets, aged 1 to 7 days, from 15 different pig farms located in a densely swine-populated area in Minas Gerais, Brazil. In each farm, samples from two diarrheic and two non-diarrheic piglets were collected (n=60). All samples were stored at 20°C and processed within 72 hours after collection.

C. difficile A/B toxins were detected using an ELISA kit (Ridascreen *Clostridium difficile* toxins A/B, R-Biopharm, Germany). The reaction was carried out in accordance to the manufacturer's instructions.

To select *C. difficile* spores, equal volumes of stool samples and ethanol 96% (v/v) were mixed, and after incubation for 20 minutes at room temperature (AVBERSEK et al., 2009), aliquots of 100µl were inoculated on plates containing cycloserine-cefoxitin fructose agar (CFFA, Hi-media, Mumbai) supplemented with 7% horse blood. These plates were incubated anaerobically at 37°C for 48 hours. All colonies with suggestive morphology, Gram stain appearance and typical horse-manure odor (FEDORKO & WILLIAMS, 1997) were collected and suspended in 400µl of sterile Milli-Q water. The DNA extraction was performed according to BAUMS et al. (2004), and samples were stored at 4°C until used in the PCR assay.

Genes encoding toxins A (*tcdA*), B (*tcdB*) and binary toxin (*cdtB*) were detected by multiplex-PCR. Briefly, 5µl of DNA extract was added to a PCR mixture containing 1.5mM MgCl₂, 50mM KCl, 10mM Tris-HCl, 200µM of each dNTP, 3.0U of *taq* Polymerase (Phonetría®, Belo Horizonte, Brazil) and the 8 primers shown at the table 1, at the concentrations given. The *tpi*-specific primers were deduced from alignments of internal fragments of the *tpi* gene, a housekeeping gene from *C. difficile*. The *tcdB*-specific primers were designed from a conserved region of the *tcdB* gene. The *tcdA*-specific primers were designed to flank the smallest of the three deletions in the 3' region of the *tcdA* gene characterized in A⁺B⁺ variant strains and generated a 369-bp fragment for A⁺B⁺ strains and a 110-bp fragment for A⁻B⁺ strains (LEMEE et al., 2004). The *cdtB*-specific primers were previously described by PERSSON et al (2008) and were designed from a conserved region of the *cdtB* gene, which is responsible for the codification of the binding component of the binary toxin from *C. difficile*. The final reaction volume was 25µl. Amplifications were carried out in a thermocycler (Thermal Cycler Px2 – Thermo Electron Corporation, Milford, USA) and consisted of a denaturation step of 5 min at 95°C, followed by 35 cycles of 30s at 95°C for denaturation, 30s at 52°C for annealing and 30s at 72°C for extension. A final extension cycle of 10 min at 72°C was also added. The positive and negative controls were a reference strain of *C. difficile* (ATCC 9689) and sterile Milli-Q water, respectively. Amplification products were visualized under UV light in a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich, Saint Louis, USA). *C. difficile* isolates were considered toxigenic based on the presence of genes encoding toxin A, B or both (RUPNIK et al., 2005).

Chi-square tests were used to evaluate possible association between dependent variables and clinical groups. P values of <0.05 were considered significant.

Table 1 - Primers used in multiplex-PCR for detection of genes encoding toxins A (*tcdA*), B (*tcdB*), binary toxin (*cdtB*) and *tpi* gene from *C. difficile*.

Gene target	Primer name	Sequence (5'-3')	Primer concentration (μM)	Amplicon size (bp)	Reference
<i>tcdA</i>	tcdA_F	AGATTCCTATATTTACATGACAATAT	1.0	365 or 110	LEMEE et al (2004)
	tcdA_R	GTATCAGGCATAAAGTAATATACTTT	1.0		
<i>tcdB</i>	tcdB_F	GGAAAAGAGAATGGTTTTATTAA	0.5	160	
	tcdB_R	ATCTTTAGTTATAACTTTGACATCTTT	0.5		
<i>tpi</i>	tpi_F	AA AGAAGCTACTAAGGGTACAAA	0.5	210	
	tpi_R	CATAATATTGGGTCTATTCCTAC	0.5		
<i>cdtB</i>	cdtB_F	TTGACCCAAAGTTGATGTCTGATTG	0.5	262	PERSSON et al (2008)
	cdtB_R	CGGATCTCTTGCTTCAGTCTTTATAG	0.5		

RESULTS

For detection of *C. difficile* A/B toxins, 10 samples (16.7%) were positive (Table 2). From these, seven were from diarrheic piglets, and three were from non-diarrheic piglets. There was no significant association between the detection of toxin A/B and the presence of diarrhea.

C. difficile was recovered from 12 of 60 (20%) stool samples. Of those, three strains were non-toxicogenic (A⁻B⁻) and nine were toxicogenic (Table 3). Among these nine strains, four were A⁺B⁺ and five were variant strains (A⁻B⁺). The binary toxin genes were detected in six (50%) strains, all of which were toxicogenic (Table 4). Considering only the farms, *C. difficile* was recovered from eight (53.3%) out of 15 visited farms.

DISCUSSION

In the present study, 16.7% of the animals were positive for *C. difficile* A/B toxins. These results are lower than those described by YAEGER et al. (2007) in which 50% (62/159) of the piglets tested positive. The significantly large percentage of positives found by those authors is likely because of the larger number of animals suspected to have *C. difficile* infection (100/129). In another similar study, the presence of toxins A

and B was observed in 58% of the piglets, however all piglets examined were with enteritis (SONGER et al., 2007).

The presence of non-diarrheic animals, although positive in the detection of toxins A and B both corroborates with YAEGER et al. (2007), which noted that was common to see piglets infected with *C. difficile* but without any clinical sign. However, those animals are positive for detection of toxins and have intestinal lesions when subjected to histopathological evaluation. It suggests that, in piglets, *C. difficile* infection could be subclinical, making difficult the perception of the disease on a farm. It is also interesting to remember that there are studies that suggest that most of the ELISA kits have a good specificity but a variable sensitivity (between 30 and 80%) when used with swine feces, which can lead to false-negative results (POST et al., 2002; SONGER et al., 2007).

No clear association between isolation of this bacteria and neonatal porcine diarrhea was detected, in agreement with previous studies in pigs (ALVAREZ-PEREZ et al., 2009; AVBERSEK et al., 2009). The isolation rate obtained (20%) was slightly lower than that reported by ALVAREZ-PEREZ et al. (2009) that recovered *C. difficile* from 25.9% of piglets, which were also one to seven days of age, from farms in Spain. In the present research, three non-toxicogenic and five variant strains were isolated from four different farms. In contrast, ALVAREZ-PEREZ et al. (2009) reported a much lower rate of non-toxicogenic and variants strains than observed in this study. Among 140 recovered strains, ALVAREZ-PEREZ et al. (2009) identified only seven non-toxicogenic (5%) strains and one variant strain (0.7%), all from the same farm.

Differences in isolation rates may result from differences in management practices, clustering of cases (ARROYO et al., 2007) and of differences in geographical distribution. A recent study also

Table 2 - Detection of toxins A and B from *C. difficile* by ELISA in stool samples from piglets in Brazil.

Piglets	-----ELISA (%)-----	
	Positives	Negatives
Diarrheic	7 (11.7)	23 (38.3)
Non-diarrheic	3 (5%)	27 (45%)
Total	10 (16.7)	50 (83.3)

Table 3 - PCR detection of *tcdA* and *tcdB* from *C. difficile* isolated from diarrheic and non-diarrheic piglets 1 to 7 days old.

Piglets	---- <i>Clostridium difficile</i> strains (%)----			
	A ⁺ B ⁺	A ⁺ B ⁻	A ⁻ B ⁻	Total
Diarrheic	3 (25)	2 (16.7)	0 (0)	5 (41.7)
Non-diarrheic	1 (8.3)	3 (25)	3 (25)	7 (58.3)
Total	4 (33.3)	5 (41.7)	3 (25)	12(100)

suggested prevalent variation in certain genotypes of *C. difficile* in different geographic regions (AVBERSEK et al., 2009). In addition, the carrier state of *C. difficile* seems to vary among asymptomatic individuals by species and within the same species, depending on age and other population characteristics (KEEL & SONGER, 2006).

According to ARROYO et al. (2007), the high frequency of variant strains may complicate the diagnosis of diarrhea due to *C. difficile* since there are several commercial ELISA kits designed to detect only toxin A, which might cause false-negative results. Additionally, variant strains have been involved in nosocomial outbreaks in humans (ALFA et al., 2000; KUIJPER et al., 2001).

In this study, all non-toxigenic strains were isolated from healthy animals, corroborating the findings of ALVAREZ-PEREZ et al. (2009). According to SONGER et al. (2007), inoculation of piglets at birth with a non-toxigenic *C. difficile* strain significantly reduced the effects of *C. difficile* infection on performance. In humans, according to KYNE et al. (2000), previous colonization by a non-toxigenic or a toxigenic strain reduces the risk of developing diarrhea associated with *C. difficile*. As a result, a non-toxigenic strain for competitive exclusion is under development for use in at-risk humans (SONGER, 2010).

Little is known about the clinical relevance and pathogenic role of CDT in *C. difficile* infections and most studies are related to human patients. The

presence of binary toxin (CDT⁺) observed in the present study was much higher (50%) than in previously reported studies. PERSSON et al. (2008), working with samples isolated from humans, reported that the prevalence of CDT⁺ strains was 26%, 97.3% of which were A⁺B⁺. ARROYO et al. (2007) found only 4% of strains were CDT⁺ in a study with diarrheic horses. AVBERSEK et al. (2009) also detected binary toxin genes in *C. difficile* strains isolated from piglets, but the CDT⁺ rate was not reported. Also, the absence of non-toxigenic strains positive for binary toxin corroborates previous studies (ARROYO et al., 2007; PERSSON et al., 2008; STUBBS et al., 2000). According to STUBBS et al. (2000), binary toxin genes are commonly observed in strains that possess some part of the pathogenicity locus containing the genes for toxins A and B. As CDT is a potent cytotoxin, GONÇALVES et al. (2004) suggested that it might prepare the way for toxins A and B. Alternatively, CDT can also act in synergy with other toxins, depolymerizing the cytoskeleton by a complementary mechanism. The high presence of CDT⁺ strains observed in the present study underlines the importance of more research to elucidate the role of CDT in neonatal colitis in piglets.

Recently, *C. difficile* has been isolated from ready-to-eat retail meats and salads. Many of these strains were of ribotypes associated with *C. difficile* infection in humans and food animals (RODRIGUEZ-PALACIOS et al., 2007; BAKRI et al., 2009; SONGER et al., 2009). It is also important to note that the two most common toxigenic ribotypes in pigs, which account for 90% and 80% of isolates, respectively, are also recognized as causes of *C. difficile* disease in humans (ARROYO et al., 2007). All these reports raise the possibility of *C. difficile* infection as a zoonotic disease, but more studies are needed for elucidation.

The results suggest the occurrence of neonatal diarrhea by *C. difficile* in farms in Brazil. Further studies could be useful to evaluate the minimum inhibitory concentrations of the most common antibiotics used on swine farms against *C. difficile* strains. This is the first study about toxin detection and isolation of *C. difficile* from piglets in Brazil.

Table 4 - Presence of binary toxin genes of *C. difficile* strains isolated from diarrheic and non-diarrheic piglets.

<i>C. difficile</i> strains	Binary toxin (%)		Total
	CDT ⁻	CDT ⁺	
A ⁺ B ⁺	3 (25)	1 (8.3)	4 (33.3)
A ⁺ B ⁻	0 (0)	5 (41.7)	5 (41.7)
A ⁻ B ⁻	3 (25)	0 (0)	3 (25)
Total	6 (50)	6 (50)	12 (100)

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