DOI: 10.5216/cab.v11i4.5081

OCURRENCE OF *Clostridium difficile* IN PIGS SUBMITTED TO ANTIBIOTIC THERAPY IN SANTA CATARINA STATE

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ABSTRACT

The *Clostridium difficile* is a Gram-positive opportunist, anaerobic, spore-forming rod found in the soil, water and enteric microbiota of many animal species. It has been described as the cause for enteritis in human beings and animals. In swine it has grown in importance due to the large number of cases of neonatal enteritis that affects the colon of one to seven-day-old piglets. This bacterium produces two kinds of toxins: A (enterotoxin) and B (cytotoxin). They have an important role in the disease's pathogenesis. Aiming at researching the presence of the bacterium in up to seven days piglets submitted to antibiotic therapy as well as the production of the toxins A and B in isolated samples, 8 collections were made in different regions of the state of Santa Catarina, totaling 490 samples of stool and retal swabs of piglets, gathered in the period of January to March 2008. The retal swabs were processed in the same day they were collected in the Microbiology Lab of CAV/UDESC and the stools were frozen at -4^{o}C in steril Ependorf so that they could be submitted to the ELISA test later. Twenty-tree colonies of *C. difficile* were isolated, but no one produced the A and B toxins according to the ELISA test. Of the 69 stools analyzed, 32 (46.37%) were positive samples, 3 (4.34%) were intermediate samples, and 34 (49.27%) were negative samples, according to the ELISA test.

KEYWORDS: Antibiotic, *Clostridium difficile*, diarrhea, swine.

OCORRÊNCIA DE *Clostridium difficile* EM LEITÕES SUBMETIDOS À ANTIBIOTICOTERAPIA NAS REGIÕES DE SANTA CATARINA

RESUMO

O *Clostridium difficile* é um bastonete oportunista Gram-positivo, anaeróbio obrigatório, formador de esporos, encontrado no solo, água e microbiota entérica de várias espécies animais. Tem sido descrito como causa de enterite em seres humanos e animais. Em suínos tem adquirido grande importância, devido ao grande número de casos de enterites neonatais que afeta o cólon de leitões entre um a sete dias de idade. Essa bactéria produz dois tipos de toxinas, A (enterotóxica) e B (citotóxica), que possuem papel importante na patogênese da doença. Com o objetivo de pesquisar a presença da bactéria em leitões com até sete dias de idade submetidos à antibioticoterapia bem como a produção das toxinas A e B nas amostras isoladas, realizaram-se oito coletas em diferentes regiões do Estado de Santa Catarina, totalizando 490 amostras de fezes e suabes retais de leitões, coletados no período de janeiro a março de 2008. Os suabes retais foram processados no mesmo dia da coleta no Laboratório de Microbiologia (CAV/UDESC) e as fezes foram congeladas a -4 ºC em ependorf estéril para posterior realização do teste de ELISA. Foram isoladas 23 amostras do *C. difficile*, sendo que nenhuma delas produziu as

toxinas A e B, pela análise do teste do ELISA. Já das 69 amostras de fezes analisadas, 32 (46,37%) foram positivas para a presença das toxinas, 3 (4,34%) intermediárias e 34 (49,27%) negativas, conforme demonstrou o teste de ELISA.

PALAVRAS-CHAVES: Antibiótico, *Clostridium difficile*, diarreia, suínos.

INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic spore-forming bacterium, found in the environment and in the intestinal tract of various mammals, birds and reptiles (NAGY & BILKEI, 2003). This agent is responsible for causing pseudomembranous colitis and diarrhea associated with antibiotic use in humans, horses, and in laboratory animals (POST et al., 2004). Recently, it was recognized as the etiologic agent responsible for causing enteritis in piglets (WATERS et al., 1998; SONGER et al., 2000).

In recent years the infection caused by *C. difficile* has gained significant importance and may be the cause of over 50% of neonatal diarrhea in piglets in the first week of life and death of up to 10% of piglets in maternity (SONGER et al., 2004). In the U.S., the toxigenic strain of *C. difficile* is considered the main agent and the only one responsible for 35% of neonatal diarrhea in addition to being found in association with other pathogens in 25% of other cases of enteritis (SONGER et al., 2007; MASARIKOVÁ et al., 2008).

The disease caused by *C. difficile* has been often linked to the use of antibiotics, which lead to a change in the enteric microbiota and creating the opportunity for colonization by the agent. When changes occur in this microbiota, antimicrobial-resistant bacteria can multiply, inhibiting the growth of beneficial bacteria in the intestinal tract and, thus favoring the growth of pathogenic bacteria such as *C. difficile* (FURTADO et al., 2005).

The piglets show moderate dyspnoea, abdominal distension and scrotal edema. Occasionally they have diarrhea, ascites, hydrothorax and breathing difficulty. High morbidity rate and mortality may occur. Inflammatory enteritis and edema of the mesocolon are macroscopic lesions, and the accumulation of neutrophils and fibrin in the lamina propria (Song et al., 2000) are observed in microscopy. *C. difficile* produces toxins A and B, which are primarily responsible for triggering the disease.

The diagnosis of infection by *C. difficile* is accomplished by detecting the toxins and isolating the bacterium in specific culture medium. The bacterium isolation by itself has limited value because the agent may be present in healthy piglets. For a definitive diagnosis, it is necessary to identify toxins A and B in stool by ELISA test (SOBESTIANSKY & BARCELLOS, 2007).

The purpose of this study was to determine in the State of Santa Catarina the occurrence of *Clostridium difficile* in piglets undergoing antibiotic therapy and check which ones produced toxins A and B by ELISA test of isolated samples.

MATERIAL AND METHODS

To carry out this study the samples were obtained, in the period from January to March 2008, from swine maternities found in the following regions of Santa Catarina: Itajai Valley (Rio do Sul and Ituporanga), Santa Catarina Midwest (Concórdia, Jaborá, Luzerna and Erval Velho) and Southern Region (Braço do Norte and São Ludgero). Stool samples and rectal swabs were randomly collected from 490 piglets with one to seven days old, treated with broad-spectrum antibiotics on the first days of life. The rectal swabs were put in Amies transport medium with charcoal, specific for anaerobic bacteria, and feces in sterile ependorf, all packed in styrofoam boxes with recyclable ice and transported to the Microbiology Laboratory of the CAV - UDESC. The rectal swabs were processed on the same day

of collection, and feces were frozen at -20°C for subsequent performance of the ELISA test for toxins A and B identification.

In the laboratory, the selective culture medium cycloserine-cefoxitin-fructose agar (CCFA), specific for *Clostridium difficile* isolation, was prepared in accordance with GEORGE (1979). The CCFA was supplemented with 0.5 mL of sodium taurocholate added after sterilization. The rectal swabs were grown by the exhaustion technique in this medium and anaerobically incubated for 48 hours at 37°C in jars with Gapak, which provided an atmosphere with the following gases: N2 (80%), CO_2 (10%) and H_2 (10%). After this period, the morphlogy of the suspicious colonies was analyzed and then the colonies were submitted to Gram staining for Gram-positive rods identification.

The isolates were biochemically confirmed by motility test, indole production and gelatin hydrolysis. Isolated colonies were incubated in the same conditions on BHI enrichment medium for 48 hours and then frozen.

ELISA test was used to qualitatively determine the production of toxins A and B by *Clostridium difficile* in stool samples and isolated colonies. The first 46 units of the ELISA kit were employed to correlate toxin production by isolated colonies with the toxins production in the respective feces. The test remainders were only used in feces analysis. The test was performed five months after the last collection in the laboratory of serology of Perdigao. RIDASCREEN® *Clostridium difficile* Toxin A/B kit from R-Biopharm company uses monoclonal antibodies against *Clostridium difficile* toxins A and B. For the results evaluation and interpretation, cutoff calculation was performed. Samples with absorbance value over 10% above the calculated cutoff were considered positive and samples over 10% below the calculated cutoff were negative, always following the manufacturer's instructions.

RESULTS AND DISCUSSION

Clostridium difficile is the primary etiologic agent of both diarrhea associated with antibiotic use and pseudomembranous colitis in humans. In pigs, this agent is responsible for causing diarrhea and death in one to seven-day-old piglets. However, there are few published data on its importance for Brazilian swine production.

In this study, 23 (4.7%) strains of *Clostridium difficile* were isolated from 490 rectal swabs. In CCFA medium, the colonies were gray, nonhemolytic, round, rough, raised and they changed the medium color as described by GEORGE (1979). The presence of Gram-positive rods was verified by Gram staining. In biochemical tests, indole was negative, bacteria were motionless and they hydrolyzed gelatin, thus characterizing *Clostridium difficile* (MERZ et al., 1994).

The results of ELISA test regarding the toxin production in feces and colonies are shown in Table 1. The ELISA kit was divided into three groups. In the first one, the production of toxins A and B by the colonies was verified. In the second one, the correlation beteween toxin production by colonies and the respective feces was observed. In the third one, only the feces were used. Of the 23 isolated samples, no toxin production was verified by the ELISA test analysis. On the other hand, of the 22 stool samples correlated with the colonies, 11 (50%) were positive. Therefore, 47 stool samples were analyzed; of these, 20 (42.55%) were positive, three (6.38%) were intermediate and 24 (51.06%) samples were negative, totaling 69 stooll samples, and 31 (44.95%) positive samples. Only 69 of 490 collected stool samples were analyzed, because the ELISA kit used had a 92 sample capacity. Stool samples were selected randomly.

In CCFA culture medium, the growth of other bacteria, which were not characteristic in the Gram staining nor in biochemical tests, was observed. The colonies of these bacteria were smaller and lacked *C. difficile* morphological characteristics.

TABLE 1. Results of toxins A and B production by ELISA in samples collected from piglets in Santa Catarina in the period from January to March 2008

	Positive	Intermediate	Negative	Total
Colonies	0	0	23	23
Colonies x feces	11	0	11	22
Feces	20	3	24	47
Total	31	3	58	92

CCFA is a selective and differential culture medium which is considered the best one for *C. difficile* isolation. *C. difficile* colonies that grow in this medium are morphologically different and they present enough fluorescent properties for a presumptive identification. CCFA comprises in its formulation the antibiotics cycloserine and cefoxitin, which partially inhibit the growth of other bacteria. The choice of these antibiotics as medium components was based on the resistance level of 16 *C. difficile* strains to cefoxitin (minimal inhibitory concentration ≥ 32μg/mL) and cycloserine (minimum inhibitory concentration ≥ 1.024 mg / mL) (GEORGE et al., 1979).

The isolation of *C. difficile* colonies is considered by many authors the best diagnosis method, although the toxins research has to be performed to differentiate toxigenic from non toxigenic strains (MERZ et al., 1994). RELLER et al. (2007) suggested that the colony is more sensitive than stool for detection of toxigenic *C. difficile* strains, but the isolation of the colony is time consuming, laborious and difficult to be carried out.

In this study, *C. difficile* was isolated from stool samples of 4.7% (23/490) of the piglets, unlike the findings by YAEGER et al. (2007), who isolated the *C. difficile* in the large intestine of 47% (61/129) of the piglets. This difference can be explained by several factors, including the dilution effects of diarrhea, isolation difficulty and long freezing time (GEORGE et al., 1979). In Brazil there are no published studies on the isolation of *C. difficile* in piglets.

In 50% (11/22) of piglets, the isolation of *C. difficile* and toxin production were negative, and none of the piglets showed positive culture and toxin production. This is due to the fact that no colonies have produced toxins. Different results were found by YAEGER et al. (2007) in the United States, where 31% (40/129) of piglets proved negative for both - culture and toxin production - and in 35% (45/129) of piglets, culture and toxin production were positive. The most probable hypotheses to explain these results regards the facts that samples can indicate the presence of non-toxigenic strains, they were frozen at -20°C or that they were thawed more than once, which can cause a quick loss of cytotoxic activity (WALTERS et al., 1998; DELMÉE et al., 2001).

The toxins of the stool samples, however, were detected in 44.95% (31/69) of piglets which showed negative isolation. These results are similar to those reported in the study by YAEGER et al. (2007), who detected toxins in the colon contents of 50% (65/129) of piglets.

The toxin detection in feces but not in isolated colonies may be related to the fact that the feces are more resistant to freezing than the colonies, because, in this study the influence of freezing was apparently lower, since toxin was detected in feces and not in colonies in 44,92 % of samples. Of 69 stool samples analyzed, 32 (46.37%) showed the toxin, demonstrating that the stool is the best material to detect them.

For the detection of A and B toxins of *C. difficile* in feces or intestinal contents of piglets, POST et al. (2002) compared the cytotoxic test in cell cultures with an ELISA assay. Of the 50 samples analyzed, 20 were positive and 24 were negative for both tests, generating a correlation of 88%. The sensitivity and specificity were 91% and 86%, respectively. Thus, ELISA was considered an appropriate method for the diagnosis of CDAD (*Clostridium difficile*-associated disease).

Similar results were found by ANDERSON (2008), who observed that the

detection of *Clostridium difficile* toxin in stool is the key to the diagnosis of CDAD in humans and animals, and ELISA test is recommended, considering it is economical and time saving.

In recently published work by LIPPKE (2008), it was reported that in litters without diarrhea coccidia (8.5%) and *C. difficile* (16.6%) were more often present, and diarrhea in piglets with the *C. difficile* was observed in 10.6%, with no significant differences in presence between case and control litters. The explanation for these results, according to YAEGER et al. (2007), is because the detection of toxins A and B do not represent a good indicator for the presence or absence of diarrhea. It has been observed in a case-control study that the majority (59%) of piglets which were positive for toxins A and B showed no diarrhea compared with piglets negative for both toxins. It is speculated that there may be some other factor, in addition to toxins A and B, which is essential for the onset of diarrhea.

No association was found between the highest amount of toxin detected in stool and diarrhea, and the types of stool were not correlated to the toxins production (LIPPKE, 2008).

All samples collected are for piglets of one to seven days old. However, stool samples positive for toxin production are from piglets up to four days. Similar data were found by LIPPKE (2008), in which, as well as beta toxin from *Clostridium perfringens*, toxins A and B to *C. difficile* are destroyed by trypsin activity, causing the occurrence of infection by that agent in the early neonatal period.

CONCLUSION

It was possible to verify the occurrence of *C. difficile* in the State of Santa Catarina, by isolating the agent from one to seven day-old-7 piglets undergoing antibiotic therapy. The selective medium cycloserine-cefoxitin fructose agar was useful for the isolation of *C. difficile*. No strain isolated from *C. difficile* produced toxins A and B, when submitted to ELISA test. It was possible to detect the presence of toxins A and B of *C. difficile* from stool and rectal swabs from piglets up to four days old.

ACKNOWLEDGEMENT

To the veterinary doctors, Priscila Koerich and João Zuffo, from PERDIGÃO S.A., Videira, SC.

REFERENCES

ANDERSON, M. A.; SONGER, J. G. Evaluation of two enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in Swine. **Veterinary Microbiology**, v. 128, p. 204-206, 2008.

DELMEÉ, M. Laboratory diagnosis of *Clostridium difficile* disease. **Clinical Microbiology and Infection**, v. 7, p. 411-416, 2001.

FURTADO, C. S. D.; KOLLER, F. L.; ASANOME; BARCELLOS, D. E. *Clostridium difficile*: um grande problema ainda pouco conhecido. **Suinocultura em Foco**, v. 5, n. 14, p. 4, 2005.

GEORGE, W. L.; SUTTER, V. L.; CITRON, D; FINEGOLD, S. F. Selective and differencial medium for isolation of *Clostridium difficile*. **Journal of Clinical Microbiology**, v. 9, n. 2, p. 214-219, 1979.

LIPPKE, R. T. **Estudo caso-controle avaliando a freqüência dos principais agentes causadores de diarreia neonatal em suínos**. 2008. 70 f. Dissertação (Mestrado de Ciências Veterinárias) – Curso de Pós-Graduação em Ciências Veterinárias na área de Medicina Veterinária Preventiva, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2008. Disponível em: <http://www.lume.ufrgs.br/>.

MASARIKOVÁ, M. *Clostridium difficile*-associated disease (CDAD) in czech piglet production. In: IPVS CONGRESS, 20., 2008, Durban. **Proceedings**… Durban, 2008, v. 3, p. 251.

MERZ, C. S.; KRAMER, C.; FORMAN, M.; GLUCK, L.; MILLS, K.; SENFT, K.; STEIMAN, I.; WALLACE, N.; CHARACHE, P. Comparison of four commercially available rapid enzyme immunoassays with cytotoxin assay for detection of *Clostridium difficile* toxin from stool specimens. **Journal of Clinical Microbiology**, v. 32, n. 5, p. 1142-1147, 1994.

NAGY, J.; BILKEI, G. Neonatal piglet losses associated with *Escherichia coli* and *Clostridium difficile* infection in a Slovakian outdoor production unit. **The Veterinary Journal**, v. 166, p. 98-100, 2003.

POST, K. W.; JOST, B. H.; SONGER, J. G. Evaluation of a test for *Clostridium difficile* toxins A and B for the diagnosis of neonatal swine enteritis. **Journal of Veterinary Diagnostic Investigation**, v. 14, p. 258-259, 2002.

RELLER, M. E.; LEMA, C. A.; PERL, T. M.; CAI, M.; ROSS, T. L.; SPECK, K. A.; CARROLL, K. C. Yield of stool culture with isolate toxin testing *versus* a two-step algorithm including stool toxin testing for detection of toxigenic *Clostridium difficile*. **Journal of Clinical Microbiology**, v. 45, n. 11, p. 3601-3605, 2007.

SOBESTIANSKY, J.; BARCELLOS, D. Bacterioses. In: BARCELLOS, D.; OLIVEIRA, S. J. **Doenças dos suínos**. 2. ed. Brasil: Cânone, 2007. p. 103-104.

SONGER, J. G.; JONES, R.; ANDERSON, M. A; BARBARA, A. J.; POST, K. W.; TRINH, H. T. Prevention of porcine *Clostridium difficile*: associated disease by competitive exclusion with nontoxigenic organisms. **Veterinary Microbiology**, v. 124, p. 358-361, 2007.

SONGER, J. G.; POST, W. P.; LARSON, D. J.; JOST, H.; GLOCK, R. D. Infection of neonatal swine with *Clostridium difficile*. **Swine Health and Production**, v. 8, n. 4, p. 185-189, 2000.

SONGER, J. G. The emergence of *Clostridium difficile* as a pathogen of food animals. **Animal Health Research Reviews**, v. 5, n. 2, p. 321-326, 2004.

WATERS, E. H.; ORR, J. P.; CLARK, E. G.; SCHAUFELE, C. M. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. **Journal of Veterinary Diagnostic Investigation**, v. 10, p. 104-108, 1998.

YAEGER, M. J.; KINYON, J. M.; SONGER, J. G. A prospective, case control study evaluating the association between *Clostridium difficile* toxins in the colon of neonatal swine and gross and microscopic lesions. **Journal of Veterinary Diagnostic Investigation**, v. 19, p. 52-59, 2007.

Protocolado em: 10 dez. 2008. Aceito em: 24 ago. 2010.