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Case Report

Neonatal diarrhea in piglets associated with *cpb-2 positive Clostridium perfringens*

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Abstract

Clostridium perfringens type A (CPA) has been recognized as one of the most important cause of neonatal diarrhea in piglets. Despite its importance, the pathogenesis of CPA-associated disease is still unclear and data regarding its occurrence in Brazil is scarce. In light of this, the aim of this study was to report a case of neonatal diarrhea in piglets by CPA encoding beta-2 toxin gene (*cpb-2)*. Three three-day-old piglets from a 2000-sow herd with history of diarrhea were necropsied and intestinal samples were collected for histology, immunohistochemistry, and feces samples were collected for bacteriologic and molecular procedures. Gross and histopathology revealed superficial necrotic enteritis associated with colonies of bacilli adhered to the exposed lamina propria. These ileal and jejunum fragments were positive for *C. perfringens* by immunohistochemistry, while anaerobic colonies were identified by PCR multiplex as CPA with the *cpb-2*. No other enteropathogen was identified from intestinal samples. The *C. perfringens* isolated strains were susceptible to penicillin, metronidazole and vancomicyn and resistant to eritromycin, enrofloxacin, oxitetracyclin and lincomycin.

Key Words: Clostridial diarrhea, beta-2 toxin, enteropathogen, enteritis, piglets

Case report

Clostridium perfringens is a gram-positive, spore-forming bacterium which is classified into five types (A-E) based on production of four major toxins: alpha, beta, epsilon and iota. *C. perfringens* type A (CPA) is the most common type and can be found widely in the environment and also in the intestinal microbiota of animals (25). In piglets, CPA is recognized as an important cause of diarrhea mainly in neonatal animals, but pathogenesis of CPA-associated disease is still unclear. Another complicating factor is the complexity of the diagnosis of CPA diarrhea, which is based on the association between the clinical signs, *post mortem* evaluation, detection of *C. perfringens* and absence of others enteropathogens. Some studies suggest the beta-2 toxin as a virulence factor of CPA, and it has been associated with the occurrence of diarrhea, so its encoding gene could be used as a virulence marker in the diagnosis of CPA-associated diarrhea in piglets (4, 9, 10).

Despite the importance of CPA as an enteropathogen in swine, data regarding its occurrence in Brazil is scarce. As a result, the aim of this study was to report a case of neonatal diarrhea in three piglets caused by *C. perfringens* type A positive for beta-2 toxin gene (*cpb-2*).

Three three–day-old piglets from different groups, from a 2000-sow herd, with history of non-responsive to antibiotics diarrhea in suckling piglets, and mortality rate of 7% in this period, were submitted to the Laboratory of Veterinary Pathology at the Universidade Federal de Minas Gerais (UFMG), Brazil. At necropsy examination, a liquid, yellowish content was observed in the intestinal lumen of the three piglets, and moderate mesocolon edema in only one of them. The small and large intestinal contents were collected from each piglet for further research and differential diagnosis of *Escherichia coli, C. perfringens*,

Clostridium difficile and Rotavirus. Samples of jejunum, ileum, cecum and colon were fixed in 10% buffered formalin, subjected to dehydration, diaphanization and paraffin inclusion. Then three-micron sections were prepared and stained by hematoxylin and eosin technique for histopathological evaluation (14), and also submitted to immunohistochemistry (IHC) technique, using a rabbit polyclonal antibody for *C. perfringens* detection (1). Evaluation of stained preparations was performed using light microscopy.

For *E. coli* isolation, samples were plated on MacConkey agar (Biobrás®, Prodimol Biotechnology) followed by incubation at 37 °C for 24 hours. Colonies considered positives on biochemical tests (17) were subjected to DNA extraction by the phenol-chloroform method (19) and submitted to a multiplex PCR for pathogenicity factor genes detection (15). Four strains, kindly provided by the Veterinary Diagnostic Laboratory at the University of Minnesota, were used as positive controls: 2568 (STb, STaP, F18 and Stx2e), 2569 (STb, LT and F4), 2570 (F6 and STaP) and 2571 (STaP, F5 and F41). Amplification products were visualized by electrophoresis in 6% polyacrylamide gel followed by silver staining (15).

For isolation of *C. perfringens*, 0.08 to 0.12 g of feces were serially diluted by factors of 10, ranging from 10^{-1} to 10^{-6}. Aliquots of 50 µl of each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, Detroit, USA) and were incubated anaerobically at 37°C for 24 hours. After incubation, characteristic colonies were collected and suspended each in 400 µl of sterile Milli-Q water. The DNA extraction was performed accordingly to Baums et al. (3), and samples were stored at 4°C until use in the PCR assay. Genes encoding *cpb-2*, enterotoxin (*cpe*) and major *C. perfringens* toxins (alpha, beta, epsilon and iota) were tested by multiplex PCR (26). The products were visualized under ultraviolet light, in a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich, Saint Louis, USA).

In addition to the isolation, the minimal inhibitory concentration (MIC) of *C. perfringens* isolated strains were determined for penicillin, metronidazole, vancomicyn, erythromycin, enrofloxacin, oxytetracycline and lincomycin, by the agar dilution method, as recommended by the CLSI (6). A sample of *Bacteroides fragillis* (ATCC 25285) was used as a control strain for the test.

For *C. difficile* toxins A/B detection in intestinal contents, two assays were used: an enzyme immunoassay kit (Ridascreen *C.difficile* toxin A/B, R-Biopharm, Darmstadt, Germany) and the detection of cytotoxic effect in Vero cells (20). For Rotavirus detection, intestinal contents were diluted in Tris-HCl $CaCl_2$ buffer (2) and RNA was extracted using the phenol-chloroform method (19). Each sample was then subjected to discontinuous polyacrylamide gel electrophoresis (PAGE; 7% running gel, 3.45% stacking gel; Sigma-Aldrich) followed by silver staining (12).

Histopathological examination revealed superficial necrosis, large amounts of bacilli in close contact with exposed lamina propria (Fig. 1A), and lymphocytic and neutrophilic infiltration in the apex of villi of the jejunum and ileum, in all three examined samples. In the IHC, bacilli immunomarked were observed associated with histopathological changes described above (Fig. 1B). Rotavirus tests were negative, whereas *E. coli* was isolated in the aerobic culture but it was negative for the virulence factors searched by PCR. Suggestive colonies of *C. perfringens* were recovered in the SPS agar from the three stool samples and further identified by PCR as CPA, positive for the *cpb-2*. These strains were susceptible to metronidazole, vancomycin and penicillin, but were resistant to erythromycin, enrofloxacin, oxytetracycline and lincomycin.

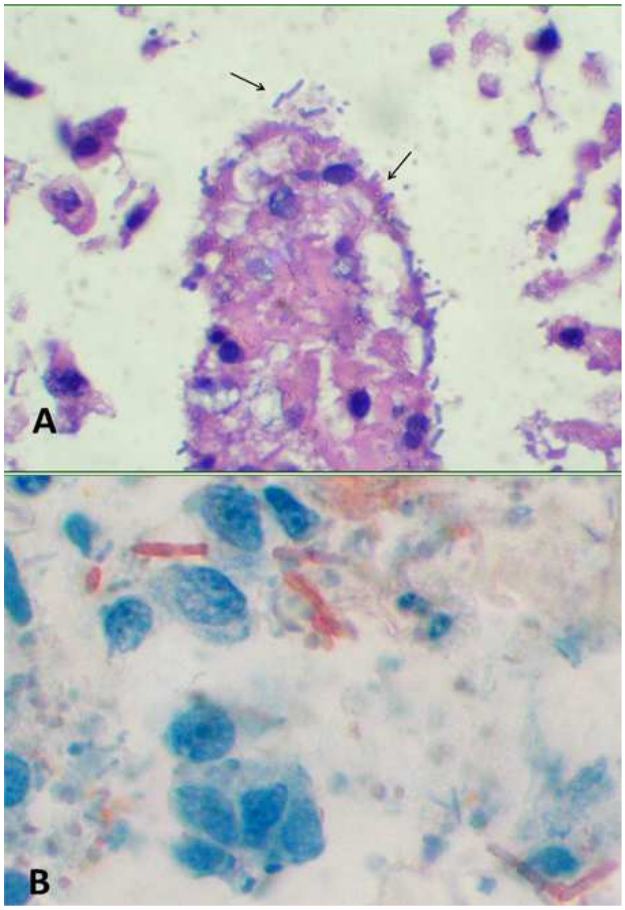

Figure 1: Swine small intestine of a piglet with diarrhea associated with *Clostridium perfringens* type A *cpb2*$^{+}$ **A –** Superficial necrosis, large amounts of bacilli (arrow) in close contact with exposed lamina propria (HE, 100X). **B** - Bacilli positive (red) for *C. perfringens* by IHC, associated with intestinal epithelium; Cromogen 3-amino-9-etilcarbazol (AEC) (100X).

Enteric disorders are the most common group of infectious diseases in piglets during the first week of life (24). They are responsible for significant economic losses, mainly due to reductions in weaning weight, increase in mortality rates and production costs (16, 24). In the present work, a high mortality rate was reported, similarly to a previous report by Costa et al. (8), but in contrast, Collins et al. (7) reported morbidity over 90%, but with low mortality rate, under 1% in a 120-sow herd with CPA-associated diarrhea.

The *post mortem* findings seen in this study are similar to those previously described (7, 11, 24). It is remarkable that some authors associate CPA *cpb-2* positive infection with the presence of inflammatory infiltrates in the lamina propria and mild to moderate necrosis of the enterocytes, but others report the absence of pathological changes, suggesting that some cases of CPA-associated enteritis may consist mainly of secretory diarrhea (11, 24).

Despite the large number of reports of CPA-associated diarrhea worldwide and its known importance for swine industry, in Brazil there are only few studies about this disease. Furthermore, most authors did not provide histological evaluation of the intestines, and sometimes differential diagnosis of other enteropathogens was not performed. These two points are considered essential to the diagnosis of CPA-associated diarrhea in neonatal piglets (8, 13, 26).

Diagnosis of CPA enteritis in piglets is complicated by the fact that differentiation between normal flora and disease-causing strains is not possible. An exception to this statement is the information indicating that most strains from diarrheic piglets are *cpb-2* positive (24, 9, 10). Based on this, the present diagnosis is supported by gross and microscopic findings, detection of a *C. perfringens* positive for *cpb-2* gene, and finally the absence of other known relevant enteropathogens. In addition, in the present work, an IHQ assay for *C. perfringens* detection was performed, which allowed to visualize a great number of antigen labeled bacilli associated with the superficial necrosis, suggesting this to be the cause of the lesions. Similarly, Collins et al. (7) reported a diagnosis a CPA infection in piglets, by the association of the absence of other enteropathogens, compatible *post mortem* evaluation and isolation of *C. perfringens*. Unfortunately, the authors give no information about detection of *cpb-2* by PCR, making comparisons difficult.

The use of autogenous toxoids for prevention of CPA-associated diarrhea has been reported in some studies (11, 23), but so far there is no evidence of efficacy in preventing disease. It is important to note that there is no known animal model to reproduce the CPA diarrhea seen in piglets, and the evaluation of a toxoid in a naturally infected herd is difficult since other management variables are often present. The most common approach to prevent CPA-associated diarrhea in a herd is the association between efforts to maximize the colostrum uptake by the piglets, and the reduction of this and other pathogens in the environment. It includes washing the sow prior to farrowing, routine disinfection of farrowing rooms and use of antimicrobials in the sow feed during pre-farrowing period. Finally, in the case of onset, injectable antibiotics are an option for piglets' treatment. Bacitracin, lincomycin and sulfa-trimethoprim are indicated, and the most commonly used in the feed, while penicillin is an injectable drug commonly used in the field (23).

In the present report, the three *C. perfringens* isolated strains were susceptible to penicillin, metronidazole and vancomicyn, corroborating previous studies that reported rare resistance to these compounds (22). Resistance to erythromycin, oxytetraciclin, enrofloxacin and lincomycin were previously described in *C. perfringens* isolates from swine and other species (18, 21, 22). For erythromicyn and oxytetracycline, the resistance is likely due to the presence of previously described genes. For erythromycin, the resistance gene encodes an enzyme called "erythromycinmethylase", whereas the oxytetracycline resistance genes encode a "ribosome protecting cytoplasmatic protein" (5). On the other hand, resistance genes studies for lincomycin and enrofloxacin are poorly reported and resistance to these antimicrobials may be due to as yet unknown genes.

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