Semina: Ciências Agrárias
ISSN: 1676-546X
semina.agrarias@uel.br
Universidade Estadual de Londrina
Brasil

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Semina: Ciências Agrárias, vol. 34, núm. 2, 2013, pp. 3923-3928
Universidade Estadual de Londrina
Londrina, Brasil

DOI: 10.5433/1679-0359.2013v34n6Supl2p3923

An outbreak of *Clostridium difficile*-associated diarrhea in piglets in Brazil

Surto de diarréia por *Clostridium difficile* em leitões no Brasil

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Abstract

In spite of the substantial role of *Clostridium difficile* in causing diarrhea in piglets, there have been few reports of the disease caused by this bacterium in Rio Grande do Sul, Brazil. In this paper, we describe an outbreak of *C. difficile*-associated diarrhea in a pig farm in Brazil. The diarrhea rate increased in piglets 1-to-7 days old from an average of 2% to approximately 20%. Necropsied piglets showed mesocolon edema, and in a histological evaluation, severe necrotizing neutrophilic colitis was observed. The intestinal contents were positive for the A/B toxins of *C. difficile* and negative for other tested enteropathogens. The association between the clinical signs, *post mortem* findings and laboratory exams confirmed the diagnosis of *C. difficile*-associated diarrhea. The present report confirms *C. difficile* as a pathogen in swine in Brazil and highlights the need for up to date routine laboratory protocols for the diagnosis of this disease in swine.

Key words: Neonatal diarrhea, colitis, enteritis

Resumo

Apesar da crescente importância de *Clostridium difficile* como causador de diarréia em leitões, são escassos os estudos e relatos da doença no Brasil. O objetivo do presente trabalho é descrever um surto de diarréia por *C. difficile* em uma granja localizada no Rio Grande do Sul, Brasil. A frequência de diarréia em leitões com até 7 dias de vida aumentou de uma média de 2% para aproximadamente 20%. Leitões necropsiados apresentavam edema de mesocólon e, na avaliação histopatológica, uma colite neutrofílica necrotizante severa foi observada. O conteúdo intestinal foi positivo para as toxinas A e B e negativo para outros patógenos pesquisados. A associação dos sinais clínicos, achados *post mortem* e resultados dos exames laboratoriais permite confirmar o diagnóstico de diarréia por *C. difficile*. O presente relato confirma *C. difficile* como um patógeno de suínos no Brasil e destaca a necessidade de inclusão desse enteropatógeno no diagnóstico laboratorial para as diarréias em leitões.

Palavras-chave: Diarréia neonatal, colite, enterite

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Recebido para publicação 11/03/13 Aprovado em 05/11/13

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. It may be responsible for 95% of all pseudomembranous colitis cases and most cases of antibiotic-associated diarrhea in humans (SCHWAN et al., 2009). In swine, it is considered to be the most important cause of diarrhea in piglets in EUA (SONGER; ANDERSON, 2006), and some reports have raised the possibility that *C. difficile* acts as a zoonotic agent (ARROYO; STAEMPFLI; WEESE, 2007).

Brazil is currently the third largest producer of pork in the world, with a flock of approximately 39 million animals (IBGE, 2010). Despite this high production, little is known about the occurrence of *C. difficile* infection in piglets. Recently, the occurrence of the disease was confirmed by survey studies (LIPPKE et al., 2011; SILVA et al., 2011); additionally, a prevalence study about the most important enteric pathogens in swine suggested that *C. difficile* is one of the main causes of neonatal diarrhea in piglets in Brazil (SILVA; GUEDES; LOBATO, 2013). Despite the increased importance of *C. difficile* as a swine pathogen, until now, there has been no description of an outbreak of the disease in piglets in Brazil. Therefore, the aim of this paper is to describe an outbreak of neonatal diarrhea caused by *C. difficile* at a pig farm in Brazil.

Case Report

The farm is located in Gaurama, Rio Grande do Sul state, Brazil. It is a large-scale commercial breed with 2000 sows and followed an all-in-all-out indoor production flow. The pigs were housed in bins that were cleaned and disinfected before the next set of pigs was placed in them.

In December of 2011, the farm owner reported an increase in the occurrence of diarrhea, mainly in 1- to 3-day-old piglets, with a reduction in weight gain but with a low mortality rate (approximately 1.5%). According to the farm records, the diarrhea rate increased in piglets 1-to-7 days old from an average of 2% to between 19 and 23% in the last three months. Despite the use of a vaccine (the alpha and beta toxins of *C. perfringens* and enterohemorrhagic *Escherichia coli*), the incidence of diarrhea did not decrease.

In the farm, 18 diarrheic and two apparently healthy piglets (n=20), from different litters and between 1 and 7 days old, were euthanized by electrocution and exsanguinated. All macroscopic lesions were recorded, and stool samples from seven animals (two apparently healthy and five diarrheic) were collected directly from the rectum and stored at 4°C for up to 48 hours. In addition, two diarrheic piglets were selected, and samples of the jejunum, ileum, cecum and colon were fixed in 10% buffered formalin for histological assays.

Stool samples were submitted to coccidian oocyst detection by the flotation method (HOFFMANN, 1987), rotavirus detection by polyacrylamide gel electrophoresis followed by silver staining (HERRING et al., 1982), isolation of *C. perfringens* and *C. difficile* (SILVA et al., 2011; VIEIRA et al., 2008) and routine bacteriologic culture for aerobic bacteria in MacConkey agar (Biobrás®, Prodimol Biotechnology) and Muller-Hinton agar supplemented with 5% of equine blood (Difco Laboratories, Detroit, USA). For A/B toxin detection, a cytotoxicity assay method (CTA) was used with African green monkey kidney cells (Vero–ATCC CCL 81) (VAN DER BERG et al., 2005).

Results and Discussion

In the *post mortem* examination, 18 out of 20 piglets (16 diarrheic and two that appeared to be healthy) had mesocolon edema. Of these, samples from two diarrheic piglets were subjected to histological evaluation, revealing severe necrotizing neutrophilic colitis with intense infiltration of neutrophils from the lamina propria to the intestinal lumen.

The parasitological exam was negative for oocysts, and only *E. coli* was obtained in the routine bacteriologic culture of the stool samples. Using a previously described PCR method for the detection of *E. coli* virulence factors (MACÊDO et al., 2007) it was determined that all isolated strains were not enterotoxigenic. *C. perfringens* type A was isolated from one diarrheic and one non-diarrheic piglet, but both strains were negative for the beta-2 toxin gene, which is a known virulence factor previously associated with *C. perfringens* diarrhea in piglets (SCHOTTE; TRUYEN; NEUBAUER, 2004).

Seven of the stool samples were positive for the A/B toxins by the cytotoxicity assay using Vero cells. In addition, *C. difficile* was isolated from three samples, two from diarrheic piglets and one from a non-diarrheic piglet, and all strains were positive by PCR for the toxin A (*tdcA*) and B (*tcdB*) genes but were negative for the binary toxin gene (*cdtB*). The minimal inhibitory concentration (MIC) of these three isolates was determined by the agar dilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). All isolates were susceptible to metronidazole, vancomycin, oxytetracycline and florfenicol, whereas the isolates showed an intermediate sensitivity to lincomycin and penicillin and resistance to erythromycin and tylosin.

In the present work, 18 out of 20 piglets, including two that appeared to be healthy, displayed a mesocolon edema, which is considered the main *post mortem* alteration associated with *C. difficile* infection (CDI) (YAEGER; KINYON; SONGER, 2007). Despite the mesocolon edema and the diarrheic content of some animals, no other relevant alterations were observed, which is common for CDI in piglets. Other reported signs, such as hydrothorax, respiratory distress, scrotal and facial edema and even sudden death, can also occur but are rare (SONGER; UZAL, 2005). In addition to this macroscopic lesion, the two diarrheic piglets that were submitted to a histological evaluation showed neutrophilic colitis, a common histological alteration attributed to *C. difficile*-associated disease (YAEGER; KINYON; SONGER, 2007).

Despite the known association between these macro- and microscopic alterations and the occurrence of CDI in piglets, these factors could not unequivocally confirm the diagnosis, which required the detection of A/B toxin, commonly performed by cell culture (the "gold-standard") or by ELISAs (DELMÉE, 2001). In some cases, the association between isolates and the detection of toxin genes may also be useful. In the present report, all of the aforementioned assays were conducted and the A/B toxins were detected by a cytotoxicity assay in all samples tested, including the two from the non-diarrheic piglets. Toxigenic strains of *C. difficile* were recovered from three animals.

It is interesting to note that three of the piglets were positive for A/B toxin detection but were negative for *C. difficile* isolation. This result corroborates previous studies with piglets and other domestic species (BÅVERUD et al., 2003; CLOOTEN et al., 2008; SILVA et al., 2011) and may be due to the susceptibility of some *C. difficile* strains to one or both of the antibiotics used in the medium (SONGER; UZAL, 2005). The detection of A/B toxins in non-diarrheic piglets is also a previously reported event. According to Yaeger, Kinyon and Songer (2007), the absence of diarrheic content in the colon does not exclude the possibility of infection, and once in piglets, the CDI commonly causes a subclinical disease.

In the present work, 18 out of 20 necropsied animals showed mesocolon edema, while all stool samples tested were positive for A/B toxins. According to Songer (2004), at farms with CDI, approximately 30% of the piglets are positive for A/B toxin, but it can reach 100% in rare cases. Considering that CDI is commonly a subclinical disease in piglets (YAEGER; KINYON; SONGER, 2007), the high incidence of diarrhea (approximately 20%) is an interesting feature of this case. It is also important to note that no other enteropathogens

were detected, and all piglets sampled were positive for A/B toxins. This high diarrhea rate, which was associated with *post mortem* alterations and laboratorial assay results, suggests an outbreak of *C. difficile* diarrhea in this farm.

Currently, CDI is considered the most important cause of diarrhea in neonatal piglets in EUA (SONGER; ANDERSON, 2006). On the other hand, little is known about the prevalence of this disease in all of Latin America. Until now, only two reports were conducted in Brazil and both showed a prevalence of approximately 16% in piglets (LIPPKE et al., 2011; SILVA et al., 2011). It is also interesting to note that, according to Silva et al. (2011), 53% of the sampled farms had at least one animal positive for A/B toxins, suggesting a high dissemination of the disease in Brazilian farms. In light of this, similar to reports in EUA and Europe, this entire work has indicated that *C. difficile* is an important enteropathogen in swine in Brazil and the need for more studies regarding the prevention and control of this disease.

All *C. difficile* isolates in the present report were susceptible to metronidazole and vancomycin, which are commonly used antibiotics for the treatment of CDI in humans and horses (BÅVERUD, 2004; SPIGAGLIA; BARBANTI; MASTRANTONIO, 2011). These results are similar to previous studies in various species (MARKS; KATHER, 2003; BÅVERUD et al., 2003; SPIGAGLIA; BARBANTI; MASTRANTONIO, 2011), and at present, metronidazole-resistant isolates have been found only in humans and horses (JANG et al., 1997; MAGDESIAN, et al. 2006). Likewise, all *C. difficile* isolates were also susceptible to oxytetracycline and florfenicol. It is also interesting to note that oxytetracycline was one of the compounds commonly used in this farm. A wide range of susceptibilities to tetracycline is commonly reported in *C. difficile* isolates from swine, but in general, nearly all isolates are susceptible (POST; SONGER, 2004). No data regarding the MIC of florfenicol were found for the *C. difficile* isolates from swine.

All of the *C. difficile* strains showed an intermediate susceptibility to penicillin and lincomycin. The resistance of human and animal *C. difficile* isolates to penicillin was also previous reported (BÅVERUD, 2004; SILVA et al., 2012; SPIGAGLIA; BARBANTI; MASTRANTONIO, 2011). It is also important to note that, according to Båverud (2002), beta-lactams are commonly implicated in *C. difficile*-associated diarrhea after antibiotic treatment in foals and horses.

The resistance to erythromycin and tylosin, both macrolides, was not a surprise and corroborates previous works with *C. difficile* isolates from other sources, including humans (DELMÉE; AVESANI, 1988; SPIGAGLIA, BARBANTI; MASTRANTONIO, 2011). It is known that *Clostridium* species can carry the *ermQ* gene, which encodes for an erythromycin resistance methylase (SLAVIĆ et al., 2011). Studies examining the antimicrobial sensitivity of *C. difficile* strains isolated from piglets suggest that tylosin, which is included in animal feed, may be useful in prophylaxis or therapy, but similar to our findings, some strains were also resistant (SONGER; ANDERSON, 2006). It is noteworthy that tylosin was also commonly used in the pigs feed of this farm. Nevertheless, the MIC results must be interpreted with caution, as most *C. difficile* isolates from cases of antimicrobial-induced CDI in humans were susceptible to the implicated drug (DZINK; BARTLETT, 1980), suggesting that successful antibiotic therapy is most likely dependent on both the susceptibility of *C. difficile* and other components of the enteric microbiota (BÅVERUD et al., 2003).

Because *C. difficile* vaccines are not currently available in Brazil, the control of *C. difficile* infections in domestic animals is based on general management measures (SILVA; GUEDES; LOBATO, 2013). Recently, some reports raised the possibility that *C. difficile* is a zoonotic agent (ARROYO; STAEMPFLI; WEESE, 2007). More studies are needed for confirmation, as, until this moment, no evidence for transmission between

humans and animals was found (MCNAMARA et al., 2011). If this suspicion is confirmed, preventing the disease (and even preventing colonization) in domestic animals should be made a priority (SILVA; GUEDES; LOBATO, 2013).

The association of the macroscopic and microscopic alterations and laboratory results confirmed the diagnosis of *C. difficile*-associated diarrhea. This report reveals the possibility of an underestimated incidence of diarrhea caused by this agent in piglets in Brazil and highlight the possibility of outbreaks of the *C. difficile*-associated diarrhea in piglets, which is consider a rare presentation of the disease.

Acknowledgments

This work was supported by funds from FAPEMIG, CAPES, INCT-Pecuária e CNPQ and PRPq-UFMG.

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