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Brief communication

Use of equine-origin antitoxins in piglets prior to exposure to mitigate the effects of *Clostridium difficile* infection – a pilot study

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Summary

Administration to newborn pigs of an oral or intraperitoneal dose of equine-origin *Clostridium difficile* antitoxin 4 hours before orogastric inoculation with a swine-origin *C difficile* field isolate resulted in lower histopathology scores 72 hours post challenge than in pigs receiving no antitoxin ($P < .05$).

Keywords: swine, *Clostridium difficile*, *Clostridium difficile*-associated disease, antitoxin, prevention

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Resumen - Uso de antitoxinas de origen equino en lechones antes de la exposición para mitigar los efectos de la infección de *Clostridium difficile* – un estudio piloto

La administración a cerdos recién nacidos de una dosis intraperitoneal u oral de la antitoxina *Clostridium difficile* de origen equino 4 horas antes de la inoculación orogástrica con un aislado de campo de *C difficile* de origen porcino resultó en índices histopatológicos más bajos 72 horas después del reto que en cerdos que no recibieron la antitoxina ($P < .05$).

Résumé - Utilisation d'antitoxine d'origine équine chez des porcelets avant l'exposition afin de limiter les effets d'une infection par *Clostridium difficile* – une étude pilote

L'administration orale ou intra-péritonéale à des porcelets nouveau-nés d'une dose d'antitoxine contre *Clostridium difficile* d'origine équine 4 heures avant l'inoculation oro-gastrique d'un isolat de *C difficile* d'origine porcine a résulté en une diminution des pointages des lésions histopathologiques 72 heures post-inoculation comparativement à des porcelets ne recevant aucune antitoxine ($P < .05$).

In the last 10 years, *Clostridium difficile* has been implicated as a major cause of neonatal diarrhea in pigs.[1] *Clostridium difficile* infection (CDI) typically affects piglets ranging in age from 1 to 7 days. Clinical signs of CDI include diarrhea, abdominal distention, and scrotal edema, with most of the pathology being attributed to toxins A and B.[2] The prevalence of *C difficile* is widespread in the United States and has been referred to as the most important uncontrolled cause of neonatal diarrhea in the pig.[1] This is supported by many studies indicating a prevalence rate of about 50% and the fact that *C difficile* may affect litter productivity by as much as 10% to 15%.[1,3,4]

In human medicine, intravenous administration of immunoglobulins for treatment of CDI has variable results.[5-8] This variability may be due to differences in timing of antibody administration and toxin exposure.[7] In a mouse model, McPherson et al[5] reported that intravenous administration of immunoglobulins is most effective when performed at the same time as toxin infusion. The use of prophylactic antibiotics has been unsatisfactory and unrewarding for swine producers.

The objective of this pilot study was to investigate if administration of an equine-origin antitoxin would serve as a beneficial intervention in minimizing the clinical and histologic effects in neonatal pigs infected with *C difficile*.

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Materials and methods

The experimental protocol was approved by the Iowa State University (ISU) Institutional Animal Care and Use Committee.

Animals and housing

Thirty-six newborn piglets were obtained from a commercial farrowing unit. Parturition was monitored on-farm and all piglets were farrowed onto a sterile drape or manually removed to prevent contact with the environment, as described by Lizer et al.[9] The piglets were immediately dried and placed in clean plastic totes under heat lamps. Colostrum was collected from farrowing sows and mixed to create a single pooled colostrum stock. All piglets were orogastrically intubated and fed 10 mL of pooled colostrum, followed by 15 mL of milk replacer (Esbilac liquid puppy formula; Pet-Ag, Hampshire, Illinois), tagged, and transported back to ISU within 4 hours of birth. Pigs were randomly assigned to six groups (Table 1) using a random number generator (Excel; Microsoft, Redmond, Washington). Inoculated pigs (Groups D, E, and F) were housed in one room while non-inoculated pigs (Groups A, B, and C) were in a separate room to prevent cross-contamination. All pigs were

Table 1: Experimental design for conventional newborn pigs receiving saline or an oral or intraperitoneal dose of equine-origin *Clostridium difficile* antitoxin and inoculated 4 hours later with sham inoculum (non-infected) or *C difficile* spores (infected)

Treatment group	Analysis group*	No. of pigs	Treatment†	Inoculation‡
A	NI	4	Saline	Sham
B	NI	4	Oral antibodies	Sham
C	NI	4	IP antibodies	Sham
D	IA	8	Oral antibodies	*C difficile*
E	IA	8	IP antibodies	*C difficile*
F	I	8	Saline	*C difficile*

* For purposes of statistical analysis, treatment groups were further categorized as NI (non-infected), IA (infected and received antibodies), or I (infected but received no antibodies).

† Treatments: either oral saline (control) or equine plasma from horses hyperimmunized against *C difficile* toxins A and B (Mg Biologics, Ames, Iowa) administered either orally or intraperitoneally (IP).

‡ Pigs were inoculated orogastrically either with sham inoculum (phosphate buffered saline; NI) or with 2×10^9 *C difficile* spores 4 hours after receiving treatment of either saline (I), or oral or IP antibodies (IA).

individually housed in raised plastic decks partitioned into individual pens (approximately 0.7 × 0.7 m) with solid dividing walls and individual feeding bowls as described by Lizer et al.[9] Pigs were fed milk replacer three times daily for the duration of the experiment (72 hours).

Study design

Pigs in groups B, C, D, and E (Table 1) received an oral or intraperitoneal (IP) dose of equine-origin *Clostridium difficile* antitoxin, and pigs in groups A and F received a saline placebo. Toxin-neutralizing antitoxin was administered at the same time as pooled colostrum. Serum samples from all pigs were tested for circulating toxin-neutralizing antibodies prior to administration of colostrum and antitoxin, and 24 hours post administration.

Inoculum

Inoculum preparation was performed as described by Lizer et al.[9] Briefly, pure pellets of *C difficile* (ISU isolate 13912–1) with a concentration of 2×10^9 spores per mL were used as the inoculum. This isolate is a 2008 field isolate from a 2-day-old scouring pig from northern Missouri that was submitted to ISU Veterinary Diagnostic Laboratory. Immediately prior to challenge, spores were heat shocked in a water bath at 80°C for 10 minutes. Brain heart infusion broth with 0.1% taurocholic acid and 5% fetal bovine serum was added to the heated spore suspension at a concentration of 25% volume per volume (v/v) and incubated 1 hour at 37°C. Sterile phosphate buffered saline was used in the place of spores for controls (sham inoculum). A 1.25-mL inoculum dose or phosphate buffered saline was administered via a sterile gastric tube and flushed with 20 mL milk replacer. Pigs were inoculated 4 hours post administration of colostrum and antitoxin or saline.

Antitoxin

Equine plasma from horses hyperimmunized against *C difficile* toxins A and B was obtained from Mg Biologics (Ames, Iowa). The hyperimmune equine plasma that was administered to the pigs had titers of 1:800 and 1:1600 for toxins A and B, respectively. Titers were determined by cell neutralization assay as described below.

Antibodies

Toxin-neutralizing antibodies were assessed in cell culture using Chinese hamster ovary cells according to the protocol established by Post et al.[10] Briefly, Chinese hamster ovary cells are exposed to dilutions of serum and known concentrations of toxins A and B. Toxin and serum are incubated for 1 hour at 37°C prior to cell exposure. Twenty-four hours later, the cells are assessed for cytopathic effect. The last dilution where no cytopathic effect is observed is reported as the antitoxin titer. The pooled colostrum sample was also tested for antibodies to toxins A and B.

Necropsy and histopathology

All pigs were euthanized by an intravenous overdose of pentobarbital at 72 hours post inoculation. At necropsy, weight, body condition (0 = normal, 1 = thin, 2 = emaciated), stomach fill (0 = empty, 1 = half full, 2 = full), consistency of large intestinal contents (0 = firm, 1 = normal, 2 = pudding-like, 3 = watery) were assessed with their respective scales, while dehydration, fecal staining of the perineum (used as a proxy for diarrhea), visible colonic necrosis and fibrin, and mesocolonic edema were assessed using a scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe) in a blinded fashion as previously described.[4,9]

Formalin-fixed tissues collected for histopathology included ileum, jejunum, descending colon, cecum, and a cross section through the spiral colon containing four to five loops. Tissues were evaluated for goblet cells, quantity of neutrophils in the lamina propria, mucosal alterations (ulcers and erosions), and mesenteritis (Lizer et al).[9]

Bacterial culture and toxin detection

After necropsy, spiral colon contents were cultured directly onto *C difficile* selective agar (CDSA; Remel, Lenexa, Kansas) in addition to routine aerobic and anaerobic plates. Toxin swabs collected from the rectum prior to inoculation and 48 and 72 hours post inoculation were assayed with a commercially available *C. difficile* Tox A/B II ELISA kit (TechLab, Blacksburg, Virginia) and analyzed on a microplate reader to grade the toxin levels on a scale from 0 through 4+ per manufacturer recommendations.

Statistical analysis

In analyzing the data, we combined scores into three general categories: clinical signs, gross lesions, and microscopic lesions. The scoring system for each category was based on that published by Lizer et al.[9] Clinical sign scores were calculated by summing scores for body condition, dehydration, and perineal staining. Gross lesions included the summed scores of necrotizing lesions, mesocolonic edema, toxigenic culture, and toxin. Microscopic lesion scores were the sum of all histopathology changes noted. For statistical analysis, pigs in groups A, B, and C were combined in Group NI (non-infected), pigs in groups D and E were combined in Group IA (infected and received antitoxin), and pigs in Group F were in Group I (infected only), as summarized in Table 1. Statistical differences ($P < .05$) in group outcomes were determined by ANOVA, Tukey's honestly significant difference (HSD) test, and Fisher's exact test using JMP Pro 10 (SAS; Cary, North Carolina) statistical software.

Results

Antibodies for toxins A and B were not detected in the pooled colostrum sample or in the serum sample from any pig prior to administration of hyperimmune equine plasma. Twenty-four hours later, all pigs that had received antitoxin either by IP or oral administration (groups B, C, D, and E) had measurable levels of circulating antitoxin. All but one pig (Group B, titer 1:2) had toxin-neutralizing titers of 1:16 or greater. Pigs that had not received antitoxin had no detectable antibodies to *C difficile* toxins 24 hours post administration of colostrum.

Clostridium difficile was isolated from the colon of all inoculated pigs at necropsy. One pig from Group A and one from Group B were culture-positive for *C difficile* at the end of the study and were excluded from all analyses. Both were from non-infected groups. Additionally, *C difficile* toxin was detected in six of the 16 pigs (37.5%) in group IA and four of the eight pigs (50.0%) in group I.

At the time of colostrum administration, mean body weight was 1.38 kg (SD 0.263). At 72 hours post challenge, the mean weights of the infected pigs (Group D, E, and F; 1.26 kg, SD 0.264) and non-infected pigs (Group A, B, and C; 1.40 kg, SD 0.329) did not differ ($P = .21$). Additionally, at necropsy, mean weights of infected pigs not receiving antitoxin (Group F; 1.21 kg, SD 0.270) and of those that did receive antitoxin (groups D and E; 1.29 kg, SD 0.265) did not differ ($P = .46$).

Results of scoring at necropsy are summarized in Table 2. There were no statistical differences in means among the groups. Two pigs in the I group and two in the IA group had mesocolonic edema. In the I group, both pigs had moderate edema, and in the IA group, one had mild and the other moderate edema. Intestinal content consistency did not differ among pigs regardless of treatment group. Gross intestinal lesions were not observed.

Microscopic lesions were summed to provide a total microscopic lesion score. Mean total scores for NI (2.90, SD 0.526) and IA pigs (3.69, SD 0.561) did not differ ($P = .86$). However, mean total score did differ between animals in Group I (7.88, SD 2.467) and either Group NI ($P = .02$) or Group IA ($P = .04$).

Discussion

Lower total microscopic lesion scores in infected pigs receiving antitoxin either orally or IP suggest a beneficial effect of administration of antitoxin prior to exposure to *C difficile*. Other parameters measured differed numerically in groups treated with antitoxin, but due to small sample sizes and wide variances in the groups they were not statistically significant. Although perineal staining did not differ among groups, it is interesting to note that all pigs from Group I had some degree of staining at necropsy, while five pigs in Group NI and five in Group IA had no staining.

Results of this pilot study also support findings by McPherson et al[5] in that intravenous administration of immunoglobulins can be effective in protecting mice when administered at the time of exposure. This intervention can easily be performed under routine swine production practices, as CDI is often predictable within a particular swine operation. Although our study size was small, there appeared to be no clinical or statistical difference in the parameters measured between pigs treated with immunoglobulins IP or orally. In routine field settings, oral administration would be simpler and less invasive for the pigs, assuming they are treated before gut closure has occurred.

In this study, we used harvested plasma containing immunoglobulins that had been specifically targeted against *C difficile* A and B toxins. Human studies[5-8] utilize immunoglobulins obtained from pooled human blood and containing antibodies to many different antigens. The ability to obtain plasma with high levels of *C difficile* A and B antitoxins maximizes the potential for effectiveness. The plasma used in this study is now available commercially (AbSolution Pg, Mg Biologics) at an approximate cost of US $0.50 per pig.

Our study was not designed to evaluate the effect of inoculation dose on CDI lesions. Prior work[11] has demonstrated that the dose of inoculum does appear to affect the severity of clinical and histopathologic lesions associated with CDI. The inoculum dose used in the present study was very high. The effectiveness of the antitoxin antibodies may even be greater under natural settings, although we did not study this.

Implications

- Lower total microscopic lesion scores in treated piglets in this study suggest beneficial effects from administration of antitoxin prior to exposure to *C difficile* in piglets.
- Under the conditions of this study, in piglets treated before gut closure occurs, oral administration of *C difficile* antitoxin may be more practical than IP administration under routine field settings.

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Conflict of interest

None reported.

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Table 2: Summary data from mean (± SE) necropsy scores for body condition, dehydration, perineal staining, and stomach fill for conventional newborn pigs in a *Clostridium difficile* antitoxin study*

	NI n = 10†	IA n = 16	I n = 8	*P*‡
Body condition	0.7 ± 0.21	0.8 ± 0.19	1.1 ± 0.23	.40
Dehydration	1.0 ± 0.33	1.1 ± 0.22	1.4 ± 0.26	.68
Perineal staining	0.8 ± 0.29	1.0 ± 0.24	1.4 ± 0.18	.38
Stomach fill	1.3 ± 0.26	1.3 ± 0.18	1.8 ± 0.16	.30

* Study design described in Table 1. All animals were euthanized 72 hours post inoculation. Scoring system: body condition, 0 = normal, 1 = thin, 2 = emaciated; dehydration and perineal staining, 0 = none, 1 = mild, 2 = moderate, 3 = severe; stomach fill, 0 = empty, 1 = half full, 2 = full as described by Yaeger et al.[4]

† Two of the original 12 NI pigs were culture-positive for *C difficile* and were excluded from analysis.

‡ Groups compared using analysis of variance with $P < .05$ considered statistically significant.

SE = standard error; NI = non-infected; IA = infected and treated with equine-origin antibodies to *C difficile*; I = infected only (no antibodies).

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