RESEARCH REPORT
SWINE HEALTH

Title: *Clostridium difficile* as a Cause of Enteritis in Neonatal Pigs, **NPB # 02-102**

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II. Objectives

(1) To define the inter- and intra-herd movement of CD
(2) To evaluate the effect of prevention and control measures on the occurrence of CDAD.

III. Progress toward meeting objectives: Work under Objective 1 is complete, and that under Objective 2 is partially complete (see Preliminary Results).

IV. Status of project in regards to timeline: With completion of the competitive exclusion work, the project is complete.

V. Modifications of project from original proposal

a. The autogenous vaccination study was conducted in North Carolina rather than Iowa.
b. The competitive exclusion study was conducted in multiple farrowing barns in a single facility in North Carolina

VI. Results

1. Defining the inter- and intra-herd movement of *CD* (complete)

Porcine strains of *C. difficile* (n = 124) have been typed by RS-PCR, which allows strain-to-strain comparison of the 16S - 23S rRNA intergenic spacer region. Briefly, DNA template from individual colonies was amplified (for 35 cycles) in a standard PCR reaction, using primers CAAGGCATCCACCGT and GAAGTCGTAACAAGG. Products were visualized after electrophoresis in a 2% agarose gel and staining with ethidium bromide. Patterns were compared and isolates differing from each other by a single band were placed into different ribotypes.

Results to date (Table 1) have revealed that a single ribotype (type A) predominates among pigs, across farms, management systems, states, and time. Isolates from calves are exclusively type A, but make up less than 5% of the isolates from humans. Thus, it appears that ribotyping will be of limited value in studying the movement of *C. difficile* in herds. On the other hand, the predominance of one type may facilitate efforts at immunoprophylaxis.

Table 1. Ribotypes of strains of *C. difficile*

Species of origin	Ribotype	Number	Percent
Pig	A	103*	83
	B	3**	2.5
	C	15	12
	D	3	2.5
Cow	A	17	100
Human	A	1	4.5
	C	1	4.5
	Other types	20	91

* One strain was nontoxigenic ** Two strains were nontoxigenic

2. Impact of CDAD prophylaxis on performance (complete)

a. Autogenous vaccination (complete). The study was conducted in a 1250 sow farrow-to-wean herd which was part of a commercial pyramidal system. Sows and gilts were of mixed breed, and breeding was by artificial insemination. CDAD had been confirmed by examination of piglets for compatible clinical signs, gross and microscopic lesions, and detection of both *C. difficile* and its toxins in feces and colonic contents.

The identity of *C. difficile* isolate 2357-01, obtained from the subject herd, was confirmed on the basis of compatible colony morphology, positivity in the L-proline aminopeptidase test (PRO disk, Remel, Inc., Lenexa, KS), and chartreuse fluorescence under long-wave ultraviolet illumination. This and other isolates were examined by a PCR assay for *tcdA* and *tcdB*, the genes for toxins A (TcdA) and B (TcdB), respectively. Toxigenicity was confirmed by enzyme immunoassay (EIA; ToxAB, Techlab, Blacksburg, VA) and by neutralizable activity of toxin B in cultures of Chinese hamster ovary (CHO) cells.

For production of the immunogens, isolate 2357-01 was cultivated in a dialysis bag suspended in brain heart infusion with 0.5% yeast extract and 0.01% cysteine (BHI). Briefly, a 2.5 ml aliquot of overnight culture in BHI was inoculated into a dialysis bag (1.5 cm diameter, 6 kDa cutoff) suspended in 4 l of BHI in a 4 l Erlenmeyer flask. After incubation at 37° C for 7 days, the contents of the dialysis bag were recovered and the liquid portion was decanted. It was then centrifuged (10,000 x g, 20 min)

and filtered (220 nm pore diameter) to remove bacterial cells. Toxin was titrated on CHO cell monolayers in 96-well plates, with endpoints expressed as the reciprocal of the highest dilution resulting in 50% cytotoxicity. Toxin was inactivated by dialysis against 0.5% formalin.

The majority of the bacterial cell mass, which was contained in a mat of cells, was homogenized (Omni-mixer, Omni International, Warrenton, VA). Ten-fold serial dilutions of the homogenate were cultured direct or after heat shock (80°C, 10 min) to detect vegetative cells and spores, respectively. No attempt was made to determine the antigenic mass of non-viable cells and bacterial products. Vegetative cells and spores were inactivated by dialysis against 0.5% formalin.

The bacterin:toxoid formulation was arbitrary and each 5 ml dose contained the equivalent of 100 cytoxicity units of toxin and 1 x 10^5 colony-forming units of vegetative cells and spores.

Animals in the principal group (n = 10 sows and 8 gilts) were vaccinated twice, at 5 weeks and 9 weeks before farrowing. Serum obtained immediately prior to vaccination, and 1 week pre-farrow, as well as colostrum obtained on the day of farrowing, were examined for antibodies capable of neutralizing TcdA and TcdB.

The number of viable piglets per litter was noted, and each newborn was inoculated orally with 1 X 106 spores of *C. difficile* in a 1 ml volume. Spores were prepared by cultivation of *C. difficile* on BHI agar with 5% citrated bovine blood, incubating for 7 days at 37°C under an atmosphere of 50:50 H_2:CO_2. Colonial growth was harvested in sterile phosphate buffered saline (0.1 M, pH 7.4, PBS), washed once, and resuspended in PBS. After incubation at 55°C for 15 min to kill vegetative cells, the preparation was chilled on ice, layered onto a 3 step Percoll gradient (80, 70, and 60% Percoll, equilibrated with PBS), and centrifuged at 3000 x g for 20 min to separate vegetative cells and cell debris from spores. The pellet contained approximately 98% spores, as determined by plating dilutions on taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA).

Piglets born to principal and control sows or gilts were inoculated orally, at birth, with 1 x 10^5 spores. A rectal swab was collected from each piglet at 3-5 days and at 10 days of age for examination for TcdB (by EIA) and *C. difficile*. Piglets were observed daily through 10 days of age, and the number of diarrheic piglets noted. At weaning (18 - 21 days of age) weights were determined and pre-weaning mortality noted.

This study has been completed. Toxin-neutralizing antibodies were not detected at any time in serum or colostrum from vaccinated or nonvaccinated sows, suggesting that toxin antigens were not presented in sufficient quantity, at an appropriate time, or in a suitable manner. Previous work demonstrated the obligate role of a humoral antitoxic response in protection against CDAD, and results of this study suggest that failed efficacy may have been due to lack of such a response in vaccinated sows.

Rectal swabs were collected from three randomly-selected piglets in each litter on day 3 after birth. EIA examination of eluates revealed that most piglets [45/54 (83.3%) from vaccinated sows and 42/54 (77.8%) from nonvaccinated sows] had TcdA and TcdB in their stools on day 3. Dead piglets were examined through weaning, and mortality was not attributed to *C. difficile*infection in any case. Given that this was a production facility, it was not possible to necropsy all surviving principal and control piglets. However, colitis was documented in the principal and control piglets which were made available to us.

Clinical findings in neonatal pigs reveal no substantial differences between piglets born to vaccinated sows and those born to nonvaccinated sows (Table 2). As noted, this may be due to inefficacy of vaccination. On the other hand, the challenge dose may have exceeded that experienced by piglets through normal exposure to the organism in the environment and in sow feces, and a potentially-beneficial immune response may have been overwhelmed. It may also be that positive effects of vaccination were not seen during the 21-day observation period, but could have been manifest later, as superior feed conversion and growth rates through the finishing period. This aspect of antiCDAD immunity warrants further study, whether in the context of autogenous toxoids or other immunoprophylactic products, but it seems nonetheless likely that improved performance should be expected in the preweaning period.

We conclude that, subject to the limitations imposed by the conditions used for this trial, effective vaccination, autogenous or otherwise, against porcine CDAD may require further attention to the amount and method of presentation of toxoid antigens.

b. Competitive exclusion by nontoxigenic strains (completed). Spore preparation was based upon strain AZ 72, which is PCR-negative for *tcdA* and *tcdB*, the genes for toxins A and B, respectively. Cultures were stored in glycerol at -80°C and were thawed on ice before use. Aliquots of culture (100 microliters) were spread uniformly over the surface of plates of brain heart infusion agar, which were then incubated at 37°C for 7 days in a humidified atmosphere of 50:50 H_2:CO_2. Each culture was then harvested into ≤ 5 ml of buffered saline, washed once, and heated to 80°C for 10 min (to eliminate vegetative cells). Viable numbers of spores were determined and aliquots of 15 ml containing 10^7 spores were stored at -80°C.

The study was conducted on a farm in North Carolina. Farm history included a 9-12 month problem with scours beginning at about 1 day of age. Pre-weaning mortality was 13-15% and survivors averaged 1.44 lbs. less at weaning than unaffected pigs. Sow and gilt litters were equally affected. Autogenous vaccination with a combination *C. perfringens* type A and *C. difficile* product failed to resolve the scour problem.

Piglets in Group 1 (farrowed by a group of 29 sows and gilts) were inoculated orally with 1 ml of a suspension of spores from a nontoxigenic strain at birth. Sows and gilts (n = 29) in Group 2 were sprayed (on perineum and teats) with the spore suspension before farrowing. Dams (n = 26) and piglets in Group 3 were not treated. Number of pigs weaned per litter and average weaning weight were not significantly different among the three groups, but *C. difficile* toxins were detected in 13 litters (24/145 piglets) in Group 2 and 14 litters (20/130 piglets) in Group 3, but in only 4 litters (5/145 piglets) in Group 1. Thus, it may be possible to prevent the effects of toxin accumulation by precolonization with a nontoxigenic strain.

VII. Discussion: Ribotyping has not proven to be of value in examining movement of *C. difficile* in and among affected herds, due to the predominance of a single ribotype. However, this ribotype is different from the dominant ribotype found in humans, thus suggesting that pig:human exchange of organisms does not occur.

Methods for immunoprophylaxis of porcine CDAD are not yet available. Autogenous vaccination is often effective in controlling enteritis caused by *C. perfringens* type A, but use of this approach for prevention of CDAD was not successful in this study. Difficulties may relate to insufficient toxoid in the immunogen.

Results of the competitive exclusion trial were encouraging. Given the number of animals included (~ 29 sows and ~ 140 piglets per group), pig-to-pig variability in weight was too great to demonstrate a

difference in weaning weights among groups. However, *C. difficile* toxins were found much less commonly in the group given an oral dose of spores of a nontoxigenic strain, and the subjective measures (how the pigs looked, including vigor and activity level) suggest that spore treatment was useful. It may be revealing to run another trial with more pigs and perhaps follow individuals through the nursery, growout, and finishing phases of production. If it were possible to produce these spores, this method should be of use to producers immediately.

VIII. Lay Interpretation:

Strains of *C. difficile* from piglets are quite uniform genetically, which suggests that a single immunoprophylactic product, when developed, will be effective across the industry. Furthermore, the genetic differences between porcine and human strains imply that porcine infections need not be considered a potential food safety problem.

Successful development of immunoprophylactic products will likely have to be based on including more toxoid in the vaccines. Autogenous vaccination may be effective (since all autogenous products are likely to be different), but there are probably significant challenges for producers of such biologicals.

Nontoxigenic strains of *C. difficile* seem to function somewhat like a probiotic; when they become established in the intestine early in life, toxigenic strains are excluded. This is in keeping with findings in hamsters and currently under development for use in humans. Competitive exclusion will need further work, but may represent a simple and cost-efficient means for lessening the impact of CDAD on pig production.