Serological evaluation of a *Clostridium perfringens* type A toxoid in a commercial swine herd

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Summary

Clostridium perfringens type A (CPA) may cause suckling piglet diarrhea, which occurs within 48 hours of birth and may last approximately 5 days. Pathological findings may be unremarkable, but CPA is usually observed in or cultured in large numbers from the intestinal lumen. Preventions and treatments for CPA enteritis are variable in efficacy. A toxoid has recently become available. In this study, the serological response to the toxoid in vaccinated and nonvaccinated gilts and their progeny was evaluated in a commercial herd with a history of CPA enteritis. A toxin-antitoxin neutralization assay, quantifying neutralizing alpha antitoxin in a live-mouse model, demonstrated a difference in geometric mean alpha antitoxin titers (expressed in international antitoxin units per mL; au per mL) (± SD) between the vaccinated (239.33 ± 55.73 au per mL) and unvaccinated gilts (139.01 ± 26.35 au per mL) ($P < .05$). Passively acquired geometric mean titers were higher ($P < .05$) in piglets 2 to 4 days of age suckling vaccinated dams (231.55 ± 111.92 au per mL) than in piglets suckling control dams (112.93 ± 113.16 au per mL). These findings demonstrate that the toxoid induces a neutralizing antitoxin which is passively transferred to suckling piglets.

Keywords: swine, *Clostridium perfringens* type A, neonatal diarrhea, alpha toxoid

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Resumen – Evaluación serológica de un toxoide de *Clostridium perfringens* tipo A en un hato comercial

El *Clostridium perfringens* tipo A (CPA por sus siglas en inglés) puede causar diarrea en lechones en la maternidad, diarrea que ocurre entre las primeras 48 horas después del nacimiento y dura aproximadamente 5 días. Los hallazgos patológicos pueden ser poco importantes, pero el CPA se observa o puede ser cultivado en grandes cantidades en el lumen intestinal. La variedad de preventivos y tratamientos contra la enteritis producida por CPA varían en su eficacia. Existe un toxoide disponible. En este estudio, se evaluó la respuesta serológica al toxoide en hembras primerizas vacunadas y no vacunadas y su progenie en un hato comercial con historia de enteritis causada por CPA. Una prueba de neutralización de toxina-antitoxina, que cuantifica la antitoxina alpha neutralizante en un modelo de ratón vivo, demostró una diferencia en el promedio geométrico del título de antitoxina alpha entre los animales vacunados (239.33 ± 55.73 au por mL) y no vacunados (139.01 ± 26.35 au por mL) ($P < .05$). El promedio geométrico de los títulos de antitoxina alpha adquiridos pasivamente en lechones de 2 a 4 días de edad fueron más altos ($P < .05$) en lechones que mamaron de madres vacunadas (231.55 ± 111.92 au por mL) que en los lechones que mamaron de madres control (112.93 ± 113.16 au por mL). Estos hallazgos demuestran que el toxoide genera una antitoxina neutralizante que se transfiere pasivamente a los lechones después de mamar.

Résumé – Évaluation sérologique d'un toxoïde de *Clostridium perfringens* type A dans un élevage porcin commercial

Clostridium perfringens de type A (CPA) peut causer une diarrhée chez des porcelets à la mamelle, se produisant dans les 48 heures suivant la naissance et durant environ 5 jours. Les trouvailles pathologiques peuvent être sans intérêts mais CPA est habituellement observé ou cultivé en grand nombre à partir de la lumière intestinale. La variété des traitements et des méthodes préventives pour l'entérite à CPA son d'efficacité variable. Un toxïde est disponible. Dans la présente étude, la réponse sérologique de cochettes vaccinées et non-vaccinées et de leur progéniture envers le toxoïde a été évaluée dans un élevage commercial avec une histoire d'entérite à CPA. Une épreuve de neutralisation de toxine à l'aide d'une antitoxine permettant de quantifier l'antitoxine alpha neutralisante dans un modèle murin vivant, a permis de démontrer la différence dans la moyenne géométrique des titres d'anticorps antitoxine alpha entre les animaux vaccinés (239.33 ± 55.73 au per mL) et les animaux non-vaccinés (139.01 ± 26.35 au per mL) ($P < .05$). La moyenne géométrique des titres d'anticorps antitoxine alpha acquis passivement chez les porcelets à la mamelle âgés de 2 à 4 jours étaient plus élevés ($P < .05$) chez les

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porcelets tétant de mères vaccinées (231.55 ± 111.92 au per mL) que les porcelets tétant des mères témoins (112.93 ± 113.16 au per mL). Ces résultats démontrent que le toxoïde induit des anticorps antitoxine qui sont transférés de manière passive aux porcelets après la tétée.

Clostridium perfringens type A (CPA) is part of the normal flora of the swine intestine,[1] but is also a cause of enteric disease worldwide.[2-6] Affected piglets develop creamy or pasty diarrhea within 48 hours of birth.[7,8] Diarrhea lasts approximately 5 days, and feces may become mucoid and sometimes pink. Upon necropsy, the small intestine is flaccid, thin-walled, and gas-filled, and contents are watery but not bloody. Mucosal inflammation is mild, rarely with adherent necrotic material. The large intestine may be distended with whitish, pasty contents, but without gross lesions. Microscopically, there may be superficial villous epithelial necrosis and accumulation of fibrin, but villi may also be normal in appearance. Capillaries may be dilated, but without hemorrhage. Lesions may be heavily colonized with gram-positive rods,[5] although it is more common to find masses of organisms in the lumen.[9]

There is little information on the role of CPA toxins in the pathogenesis of CPA enteritis in piglets. *Clostridium perfringens* type A produces only one major toxin type (alpha toxin). No consistent changes occur in gut loops inoculated with purified CPA, but slight villous edema occurs in 6-hour-old piglets challenged orally or intragastrically.[1] Recently, it has been suggested that the minor toxin, beta2 (CPB2), is involved in CPA enteritis.[10,11] Its specific role in pathogenesis is unknown, but the presence of the gene coding for CPB2 production is strongly associated with piglet enteric disease.[7,12] The roles of the alpha and CPB2 toxins are not fully understood. A recent report suggests that CPB2 is implicated in the pathogenesis of enteritis in piglets.[13] In a preliminary study, piglets suckling gilts vaccinated with a CPA bacterin toxoid were protected when challenged intravenously with concentrated alpha toxin (Michelle Walz, unpublished data, 2004). However, intravenous administration of toxin is not an acceptable challenge method to demonstrate efficacy of a vaccine for product licensure in the United States.[14] The Animal and Plant Health Inspection Agency Center for Veterinary Biologics (APHIS CVB) has established antibody response levels to estimate efficacy of CPA toxoids, using a toxin-antitoxin neutralization assay (TANA). A TANA antitoxin titer < 1 international antitoxin unit per mL (au per mL) indicates a negative result and a titer > 4 au per mL is considered a positive result.[14,15] However, the TANA titer is not an indicator of "protection" in piglets. Antitoxin titers generally increase with age until they become positive in young adults, suggesting environmental exposure (Michelle Walz, unpublished data, 2004).

Various management interventions for CPA enteritis have been applied to prevent or alleviate the clinical signs of diarrhea and suboptimal weaning weight in piglets, meeting with variable success.[16] Recently, a CPA alpha toxoid has received a conditional license on the basis of a reasonable expectation of efficacy and potency.[14] This vaccine offers another option for use in CPA enteritis control programs. Because it contains only alpha toxoid, use of this CPA alpha toxoid in the field can help evaluate the clinical relevance of alpha toxin in CPA enteritis.

The objective of the study was to evaluate the antitoxin response of the conditionally licensed CPA alpha toxoid in a commercial pig herd. Seroconversion in vaccinated and nonvaccinated gilts and their piglets was evaluated using a TANA.

Material and methods

Study herd, housing, and management

A 3850-sow farrow-to-wean herd with a history of CPA neonatal diarrhea was chosen. Although CPA was not the only neonatal disease diagnosed, it was the most common diagnosis, according to the herd veterinarian. During the year preceding the study, colibacillosis and *Clostridium perfringens* Type A were identified concurrently and diagnosed twice, and coccidiosis in older piglets (14 to 20 days of age) was diagnosed once.

The farrowing facilities were divided into two sites, with 10 and 12 farrowing rooms, respectively, containing 10 to 24 crates each. Floor type varied, including wire, steel, plastic-coated wire, and concrete. Personnel moved between the sites, but at the outside door of each building, changed to footwear dedicated for inside barn use. Personal vehicles were not allowed on the farm premises.

Farrowing rooms were managed all-in, all-out, and farrowing crates were washed with a pressure washer and disinfected with a commercially available quaternary ammonium chloride and glutaraldehyde disinfectant between farrowing groups.

Pigs were weaned at an average age of 19 days. Incoming breeding stock were exposed to mummified fetuses and farrowing room feces prior to breeding, but pregnant sows were not exposed either to mummified fetuses or farrowing room feces.

Sows and gilts were bred in crates and remained in them until day 50 of gestation, when they were transferred to gestation pens (approximately 15 pregnant animals per pen). The herd was managed using the animal welfare guidelines of the National Pork Board.

Study groups

At the time of the study, a safety study was being conducted in this herd for conditional licensure of the CPA alpha toxoid. Approximately 350 randomly selected sows or gilts each received two doses of the subsequently conditionally licensed CPA toxoid, administered intramuscularly in the neck at an interval of approximately 3 weeks. A subset of 25 gilts from the safety trial, all due to farrow the same week and housed in two adjacent gestation pens, were selected by convenience for serological evaluation. These gilts were assigned by pen to two treatment groups: vaccinates (n = 12 of 350) and nonvaccinated controls (n = 13 of 42). Nonvaccinated controls were not sham-vaccinated. Piglets were not cross-fostered between vaccinated and nonvaccinated gilts. Because of the logistics of managing the cross-fostering, farm personnel were not blinded to the treatments.

Vaccination and blood sampling

Blood samples were collected from all 25 pregnant gilts the day before they received the initial dose of CPA alpha toxoid. The vaccinated gilts received two doses of the subsequently conditionally licensed CPA toxoid approximately 6 and 3 weeks before farrowing. The gilts were also vaccinated 1

week after the initial CPA toxoid vaccination with a commercial combination *Clostridium perfringens* type C-*Escherichia coli* vaccine, a commercial swine influenza vaccine containing types H3N2 and H1N1, and a commercial parvovirus, 5-way *Leptospira* vaccine. Fifteen days after the second CPA vaccination (approximately 1 week prefarrowing), a second blood sample was collected from the gilts. Blood samples were collected from all piglets at 2 to 4 days of age to assess the passive transfer of antibodies to alpha toxin (antitoxin).

Serological testing

Sera were tested for alpha toxin antibodies using a TANA based on a supplemental assay method for potency testing *Clostridium perfringens* type C beta antigen.[17,18] This TANA assay tests for actively neutralizing antibodies using mice as the indicator. Non-neutralizing antibodies are not quantified by the assay, as alpha toxin is lethal to mice at the dose administered. Standard antitoxin and standard toxin reagents used in the assay were acquired from APHIS CVB in Ames, Iowa.[17,18] The standard antitoxin is diluted to contain 1 au per mL (International Reference Preparation [IRP] 426 provided by APHIS CVB). [17] The standard toxin is also titrated to one L_0 dose and one L_+ dose (IRP 446 provided by APHIS CVB).[19] One L_0 dose of toxin is defined as the largest amount of toxin that can be combined with 1 unit of the standard antitoxin and not cause death when injected into mice. One L_+ dose of toxin is defined as the smallest amount of toxin that can be combined with 1 unit of the standard antitoxin and cause death when injected into mice. Mice used in the testing were cared for and housed according to Novartis animal welfare guidelines.

Non-serial dilutions of sera with peptone were made to estimate the antitoxin titer. Each diluted sample was then combined with an L_0 dose of toxin and controlled with the diluted standard antitoxin combined with an L_0 dose and an L+ dose of toxin to ensure the validity of the assay and determine the dilution of the sera which protected the mice. For example, to test for 50 au per mL, the serum is diluted 1:50. The diluted serum is then combined with an L_0 dose of standard toxin and injected into mice.

Two mice were injected for every serum dilution tested. Prevaccination sera were tested at dilutions of 1:4 to 1:75 for a maximum of five and minimum of two dilutions per sample, depending on the endpoint. The endpoint prevaccination dilution was used as the starting point for the postvaccination dilutions. For piglet pooled samples, doubling dilutions from 1:50 to endpoint were performed. This approach minimized the number of mice used, the number of tests performed, and use of reagents. A total of 362 dilutions (724 mice) were used in the study.

Sera from all piglets within a litter were pooled and each pool was tested for alpha antitoxin using the TANA described above.

Statistical analysis

Statistical analysis was performed using the Wilcoxon two-sample test, comparing antitoxin titers of gilt prevaccination and postvaccination samples and pooled piglet samples for the vaccinated and nonvaccinated groups. A nonparametric analysis of the results was used, which made no assumptions about the underlying distribution of the data.

Results

Serological findings are summarized in Table 1. Prevaccination titers of vaccinated and nonvaccinated groups did not differ ($P = .14$). The lowest antitoxin titer among the prevaccination samples was 4, which is considered positive, ie, indicates that the gilt has made an antibody response to alpha toxin. Postvaccination titers of vaccinated gilts were greater than those of nonvaccinated gilts, and titers of piglets born to vaccinated gilts were greater than the titers of piglets born to nonvaccinated gilts (Table 1).

Discussion

The major toxin produced by *Clostridium perfringens* type A is alpha toxin. Increasing alpha antitoxin titer in pigs to aid in the control of CPA enteritis will help evaluate the influence of CPA alpha toxin in disease pathogenesis. The CPA toxoid used in this study does not include CPB2 toxoid. An assay method to evaluate CPB2 antitoxin levels has not been developed, although several authors have indicated that CPB2 might be important in pathogenesis of CPA enteritis.[10,11,13,18,20,21] However, definitive evidence is still lacking concerning the role of CPB2 in CPA enteritis in the piglet. Other reports indicate that CPB2 genotypes are common, but expression may be variable.[10,21]

The TANA used in this study provides an antitoxin titer range, rather than a definitive value, and the lower values of the ranges were used in the statistical analysis. Therefore, the antitoxin titers reported may be assumed to be minimum values.

Table 1: Serological results of *Clostridium perfringens* type A (CPA) toxin neutralization tests performed on samples collected from vaccinated and nonvaccinated gilts on the day of the first CPA toxoid injection and 15 days after the second injection, and from their 2- to 4-day-old litters*

	Antitoxin titers (au/mL)†		
Treatment	**Prevaccination**	**Postvaccination**	**Pooled piglet samples**
Vaccinated (n = 12)	23.28 ± 20.35[a]	239.33 ± 55.75[a]	231.55 ± 111.92[a]
Control (n = 13)	11.58 ± 8.89[a]	139.01 ± 26.35[b]	112.93 ± 113.16[b]

* Gilts were vaccinated at approximately 10 and 13 weeks of gestation with a conditionally licensed CPA toxoid. Blood samples from each litter were pooled before testing. Antitoxin titers of gilt and pooled piglet samples were compared for the vaccinated and nonvaccinated groups.

† Geometric mean ± SD of titers. Samples were tested using a toxin-antitoxin neutralization assay (TANA) and reporting the lower assay point. A TANA antitoxin titer < 1 international antitoxin unit/mL (au/mL) indicates a negative result, and a titer > 4 au/mL is considered positive.

[ab] Values with different superscripts within a column differ significantly ($P < .05$; Wilcoxon two-sample test).

APHIS CVB set a reasonable expectation that achieving 4 alpha au per mL in vaccinated pigs that had been seronegative (< 1 au per mL) before vaccination demonstrates efficacy.[14] It may be noted that none of the gilts in this study were seronegative at the prevaccination sampling (using the stated cutoffs for negative samples). However, gilts vaccinated with the conditionally licensed CPA alpha toxoid and subsequently their litters did have significantly higher titers than the control gilts. The full clinical significance of alpha antitoxin levels cannot be determined until an acceptable challenge model has been developed that reflects the pathogenesis of the disease in the field, or until adequate field efficacy evaluations are performed.

Implications

- Under the conditions of this study, alpha toxin antibody titers (alpha antitoxin titers) are higher in vaccinated gilts and their piglets than in nonvaccinated gilts and their piglets.
- More field experience will be necessary to determine the clinical impact of the CPA toxoid on CPA enteritis in piglets.

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References

1. Mansson I, Smith LDS. Atypical strains of *Clostridium perfringens* from swine. *Acta Path Microbiol Scand*. 1962;55:342–348.

2. Amtsberg GW, Bisping W, el-Sulkhon SN, Matthiesen I, Krobischi P. *Clostridium perfringens* type A infection. *Berliner und Munchener Tierarzliche Wochenschrift*. 1976;21:409–414.

3. Jestin A, Popoff MR, Mahe S. Epizootiologic investigations of a diarrheic syndrome in fattening pigs. *Am J Vet Res.* 1985;46:2149–2151.

4. Nabuurs MJA, Haagsma J, van der Molen EJ, van der Heijden PJ. Diarrhea in one to three week-old piglets associated with *Clostridium perfringens* type A. *Annales de Recherches Veterinaires.* 1983;14:408–411.

5. Ramisse J, Brement A, Poirier J, Rabreaud C, Simonnet P. Flore microbienne isolae au cours de diarrhees neonatalesmortelles chez le veau, lagneau et le porcelet. *Rev Vet Med*. 1979;130:111–122.

6. Secasiu V. Diagnosticul bacteriologic welchiozel porcine(enterotoxiema anaeroba). *Revista de cresterea animalelor*. 1984;2:38–45.

7. Johannsen U, Arnold P, Kohler B, Selbitz HJ. Untersuchungen zur experimentellen *Clostridium perfringens* typ A enterotoxaemie der Saugferkel. *Monatsh fur Veterinaermed.* 1993;48:129–136.

8. Johannsen U, Menger S, Arnold P, Kohler B, Selbitz HJ. Untersuchungen zur experimentellen *Clostridium perfringens* typ A enterotoxaemie der Saugferkel. *Monatsh fur Veterinaermed*. 1993;48:267–273.

9. Johannsen U, Menger S, Arnold P, Kohler B, Selbitz HJ. Untersuchungen zur experimentellen *Clostridium perfringens* typ A enterotoxaemie der Saugferkel: Licht und elektronmikroscopischen Untersuchungen zur Pathologie und Pathogenesis der experimentellen *Clostridium perfringens* typ A Infektion. *Monatsh fur Veterinaermed*. 1993;48:299–306.

10. Bueschel DM, Jost BH, Billington SJ, Trinh HT, Songer JG. Prevalence of *cpb2*, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol.* 2003;94:121–129.

11. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer JG, Titball RW, McClane BA, Sarker MR. Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J Clin Microbiol.* 2003;41:3584–3591.

12. Gibert M, Jolivet-Renaud C, Popoff MR. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. *Gene*. 1997;203:65–73.

13. Hendriksen SW, van Leengoed LA, Roest HI, van Nes A. Neonatal diarrhoea in pigs: alpha- and beta2-toxin produced by *Clostridium perfringens*. *Tijdschr Diergeneeskd*. 2006;131:910–913.

14. United States Department of Agriculture. Center for Veterinary Biologics Notice 02-25. Conditional Licenses for Products Containing *Clostridium perfringens* Type A 2005. Available at: **http://www.aphis.usda.gov/vs/cvb/notices/2002/02-25.pdf**. Accessed 15 August 2007.

15. United States Department of Agriculture Center for Veterinary Biologics Testing Protocol. Supplemental Assay Method for Potency Testing *Clostridium perfringens* type C Beta Antigen. Available at: **http://www.aphis.usda.gov/animal_health/vet_biologics/publications/201.pdf**. Accessed 20 June 2007.

*16. Baker SR. *Clostridium perfringens* type A: Associated pathogens, lesions, control methods, and shedding. *Proc IPVS.* Copenhagen, Denmark. 2006;146.

17. Center for Veterinary Biologics. IRP 426 *Clostridium perfringens* Type A antitoxin specification sheet received June 22, 2006.

18. Vilei E, Schlatter Y, Perreten V, Straub R, Popoff HR, Gibert M, Grone A, Frey J. Antibiotic-induced expression of a cryptic *cpb2* gene in equine *cpb2*-toxigenic *Clostridium perfringens*. *Mol Microbiol*. 2005;57:1570–1581.

19. Center for Veterinary Biologics. IRP 446 *Clostridium perfringens* Type A toxin specification sheet received June 22, 2006.

20. Songer JG, Uzal FA. Clostridial enteric infections in pigs. *J Vet Diagn Invest*. 2005;17:528–536.

21. Klaasen HLBM, Molkenboer MJCH, Bakker J, Miserez R, Hani H, Frey J, Popoff MR, van den Bosch JF. Detection of beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in The Netherlands and Switzerland. *FEMS Immunol Med Microbiol*. 1999;24:325–332.

* Non-refereed reference.