PEER REVIEWED

Failure of a novel surface polysaccharide-targeting vaccine to prevent *Tritrichomonas* infection in beef cattle

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Abstract

Tritrichomonas foetus (*T. foetus*) is the causative agent of bovine trichomoniasis that has a major impact on production costs for beef cattle producers. Immunization strategies to effectively protect against *T. foetus* are a high priority. *Tritrichomonas foetus* expresses a surface polysaccharide, beta 1-6 poly-*N*-acetyl glucosamine (PNAG), that is synthesized by a broad range of microbial pathogens. Vaccination with a PNAG-specific vaccine has demonstrated protection in pigs and horses.[3,4,5] This study attempted to protect pregnant cows from an experimental *T. foetus* infection by prior vaccination with a PNAG-specific vaccine. Acceptable PNAG-specific antibody titers were achieved after vaccination, but antibodies elicited were non-protective against experimental challenge with *T. foetus* and in *in vitro* antibody functional bacterial killing assays.

Key words: trichomonas, infertility, abortion, vaccine

Introduction

Tritrichomonas foetus (*T. foetus*) is a flagellated, obligate anaerobic protozoan. This protozoan is the causative agent of bovine trichomoniasis that negatively impacts production costs for beef cattle producers.[6] A reduction in calf crop percentages by a factor of 30% is typical, and additionally, because infected cows become pregnant much later in the breeding season, smaller calves are weaned.[3] Longer breeding seasons result in longer calving seasons with reductions in overall weaning weights. Economic losses stem from 2 sources: reduced pounds of calves sold and costs associated with culling and replacing bulls. Researchers at Texas A&M University have shown that these losses amount to an estimated $156 million annually in Texas.[3] Current whole killed organism vaccines have low effectiveness in bulls and cows, thus there exists a tremendous opportunity for the development of an effective vaccine.

T. foetus expresses a surface polysaccharide, beta 1-6 poly-*N*-acetyl glucosamine (PNAG), which is synthesized by a broad range of microbial pathogens.[1,5] PNAG is expressed by the pathogen in adverse conditions, such as inflammation, and therefore thought to protect the pathogen from adverse environmental and immune environments.[1,5] PNAG has high potential to be the target of a vaccine protective against a diverse array of pathogens. However, specific glycoforms of PNAG, notably ones lacking acetate substituents on the amino groups of the glucosamine monosaccharides, are needed to produce optimal immunity.[1,5] Naturally occurring PNAG does not elicit protective antibodies.[1,5] A synthetic non-acetylated oligosaccharide-tetanus toxoid vaccine has proven safe and immunogenic in goats, rabbits, mice, pigs, dogs, cats, horses, and humans.[1,2,5] Thus far, this PNAG vaccine has been effective in protecting against *A. pleuropneumoniae* infections in pigs, *Rhodococcus equi* in horse foals, and *E. coli* K1 meningitis, *K. pneumonia*, *S. aureus*, Group A Streptococcus, *Streptococcus pseudointermedius* among others in mice.[1,2,5]

Vaccination of beef cows against PNAG may protect against *T. foetus* colonization of an infected cow's uterus and thus prevent early abortions. To investigate this hypothesis, we conducted a randomized double-blind vaccination trial and characterized the pregnancy status from embryonic attachment (~ d 30) until 4 mo of pregnancy.

Materials and Methods

Vaccine

The PNAG targeting vaccine is composed of a synthetic pentamer of glucosamine residues ($5GlcNH_2$) conjugated to tetanus toxoid (TT; $5GlcNH_2$-TT) mixed together with the water-in-oil adjuvant Speco.[a,2] The vaccine (AV0328) was provided by Alopexx Vaccines.[b] Cows in the vaccine group

received 125 µg (during 2015 for safety and immunogenicity study) or 200 µg (2016 for challenge study) of synthetic pentamers of β 1→6-linked glucosamine conjugated to tetanus toxoid (ratio of oligosaccharide to protein 35-39:1) diluted to 900 µl in sterile medical grade physiological saline (*i.e.*, 0.9% NaCl) solution (PSS) combined with 100 µl of Specol.

PNAG Expression by Clinical Isolates of T. foetus

Clinical isolates of *T. foetus* were obtained from the Bovine Infectious Disease Laboratory, Texas A&M University College of Veterinary Medicine & Biomedical Sciences. All strains were originally clinical isolates derived from infected bulls. *Tritrichomonas foetus* isolates were spotted directly onto glass slides, air dried and fixed by exposure for 1 min to undilute methanol at 39.2°F (4°C). Samples were labeled with either 5 µl of a 5.2 µg/mL concentration of human IgG1 monoclonal antibody (MAb) F598 to PNAG directly conjugated to Alexa Fluor 488[c] or control human IgG1 MAb F429 that binds to *Pseudomonas aeruginosa* alginate, also directly conjugated to Alexa Fluor 488,[c] for 4 h at room temperature. During the last 5 min of incubation, 500 nM of Syto83[c] in 0.5% BSA/PBS pH 7.4 was added to stain nucleic acids (red fluorophore). Samples were washed and mounted for immunofluorescent microscopic examination as described.[2]

Safety and Immunogenicity Studies

For the initial safety study, 6 cows were immunized with 125 ug of PNAG vaccine with 0.1 mL of Specol twice, 2 weeks apart. Vaccination was performed subcutaneously in the neck. Blood was collected prior to vaccination and at d 14, 21, and 35 post-vaccination by venipuncture. Serum was prepared from blood by centrifuging at 2,500 rpm for 15 min and then stored at -112°F (-80°C).

To assess the immunogenicity of the vaccine formulation, antibody titers to PNAG were assessed by ELISA on d 14, 21, and 35 post-immunization. Total IgG, and IgG1 and IgG2 were assessed by the following method. Briefly, ELISA plates were coated with 0.6 ug/mL purified PNAG obtained from *S. aureus* in 0.04M phosphate buffer overnight at 39.2°F (4°C). Plates were washed and blocked with phosphate buffered saline (PBS) containing 2.5% bovine serum albumin (BSA) for 1 h at 98.6°F (37°C). Plates were washed with PBS containing 0.05% Tween-20 3 times and incubated with various dilutions of control or experimental cow serum in PBS with 1% BSA for 1 h at 98.6°F (37°C). After washing, plates were incubated with either rabbit anti-cow IgG (whole molecule) conjugated to alkaline phosphatase[d] or mouse MAbs to cow IgG1 or cow IgG2[e] for 1 h at room temperature. Plates were developed using 1 ug/mL solution of pNPP[d] at 98.6°F (37°C) and optical density read at an absorbance of 405 nm.[1]

To determine the functional activity of cow antibody to PNAG, we measured the deposition of complement component C1q from both human (not shown) and cow sera as described.[1,2] Briefly, ELISA plates were sensitized with PNAG and dilutions of control and vaccinated cow sera were added in 50 µl volumes with 50 µl of 10% intact pooled cow serum as the complement source.[f] After 60 min of incubation at 98.6°F (37°C), plates were washed and goat anti-human C1q[g] that also binds to bovine C1q was added, and plates incubated at room temperature for 60 min. After washing, rabbit anti-goat IgG whole molecule[d] conjugated to alkaline phosphatase was added for 1 h incubation at room temperature. Plates were developed using a 1 ug/mL solution of pNPP[d] at 98.6°F (37°C) and optical density read at an absorbance of 405 nm.

Bactericidal assays against *Acinetobacter baumannii* and opsonophagocytic killing assays against *Staphylococcus aureus* (both are microbes that produce PNAG) were performed to further assess the functional capacity of the vaccine-induced cow antibodies. Assays were performed as described previously.[1] Briefly, for bactericidal assays, target bacteria were grown overnight at 98.6°F (37°C) on agar plates and killing assessed by incubating 2×10^5 cfu of bacteria with a 10% final concentration of human complement absorbed with the target-strain and the vaccinated cow pre-immune or immune sera at various dilutions. Tubes were incubated with end-over-end rotation for 90 min, then diluted in TSB with 0.1% proteose peptone and plated for bacterial enumeration.

To determine opsonic killing of *S. aureus,* bacterial cultures were routinely grown overnight at 98.6°F (37°C) on blood-agar plates, then killing assessed using modifications of previously described protocols.[1] Modifications included use of EasySep™ Human Neutrophil Isolation Kits[h] to purify PMN from blood, and use of gelatin-veronal buffer supplemented with Mg^{++} and Ca^{++}[i] as the diluent for all assay components. Final assay tubes contained, in a 400 µl volume, 2×10^5 human PMN, 10% (final concentration) *S. aureus*-absorbed human serum as a complement source, 2×10^5 *S. aureus* cells and the serum dilutions. Tubes were incubated with end-over-end rotation for 90 min then diluted in TSB with 0.05% Tween and plated for bacterial enumeration.

Cow Studies

Vaccination of Cows

Sixteen post-pubertal virgin heifers were used. Vaccination was performed by subcutaneously injecting 200 ug of the PNAG vaccine with 10% Specol in a 1 mL volume. A placebo was also prepared, and heifers were randomly assigned to receive either the vaccine or placebo. The injections were given twice at 2-week intervals.

Insemination and Infection with T. foetus

The estrus cycles of the heifers were synchronized by using 3 doses of 25 mg of dinoprost tromethamine[j] at 14 d intervals. Sixty h after the third dose, the heifers were inseminated with fertile frozen/thawed semen. The semen was deposited at the internal cervical os. The heifers were then immediately inoculated with 10^6 *T. foetus* organisms into the anterior vagina beneath the external cervical os. Next, the heifers were injected with 100 mcg gonadorelin[k]

to induce ovulation. All injections were delivered subcutaneously in the neck.

Evaluation of pregnancy and T. foetus infection status of vaccinated cows

The investigator performing the cultures and pregnancy testing (JAT) was blind to the heifers' vaccination status. To evaluate infection status, vaginal mucus was aspirated from the anterior vagina using a 10 mL syringe and infusion pipette. The collection was repeated at weekly intervals from d 4 post-insemination until d 32 post-insemination. The mucus was immediately inoculated into an Inpouch™[l] culture container and cultured at 98.6°F (37°C). On d 32 post-insemination the heifers were evaluated for pregnancy by trans-rectal ultrasonography. From d 32 post-insemination until 4 mo post-insemination, pregnancy evaluation was repeated by trans-rectal palpation and vaginal cultures were collected at monthly intervals. All cultures were read on d 1 to 4 and d 7 before being discarded. The organism was identified by its characteristic morphology and motility. The analysis used Fisher's Exact Test to compare proportions on infected and non-infected animals in the control and vaccine groups, respectively.

Results and Discussion

T. foetus expresses PNAG in vitro

Using immunofluorescence microscopy, we demonstrated that *T. foetus* expresses PNAG *in vitro* (Figure 1).

PNAG vaccine is safe and elicits IgG titers in cows

No swelling or adverse reactions were observed at the injection sites at any time during the vaccination process, suggesting that the vaccine formulation had no local effects on the cows and was safe to administer, as previously observed with horses, pigs, goats, and humans (data not shown).

Of the 6 cows immunized with 125 ug of the PNAG vaccine containing 0.1 mL Specol in the safety study, 4 developed titers above pre-immune sera to native (i.e., acetylated) PNAG in response to the vaccine. The remaining 2 cows made lower responses, suggesting that a higher dose than 125 ug of PNAG vaccine may improve the antibody response (data not shown). Similarly, of the 8 cows immunized with 200 ug of the PNAG vaccine containing 0.1 mL Specol in the challenge study, all 8 developed statistically significant titers to native PNAG and had no adverse reactions as determined by injection site swelling or elevated rectal temperature (Figure 2).

PNAG vaccine does not protect against T. foetus infection or impact pregnancy status

Four d post-inoculation of *T. foetus* into the vagina, all 16 heifers cultured positive for *T. foetus*. Organisms were so plentiful that all 16 cultures were determined to be positive immediately after inoculation. All cultures were again positive on d 11 post-inoculation. One heifer in the control group was culture negative starting at d 18 post-inoculation (approximately the time of embryonic signaling). All other heifers (15/16) remained positive on vaginal culture at least until pregnancy testing on d 32 post-insemination. On d 60, 14/16 heifers were culture positive and on d 90, 5/16 heifers were culture positive. At the conclusion of the study (d 120), 3/16 animals were culture positive, including 1 pregnant cow in the control group and 2 non-pregnant cows in the vaccinated group. The rates of positive culture were not significantly different between vaccinates and controls ($P > 0.1$).

With ultrasound pregnancy testing at d 32 post-insemination, 3 heifers in the control group were pregnant and 2 cows in the vaccinated group were pregnant. One cow in the vaccinated group aborted before 4 mo of gestation, as determined by abortion diagnostics, leaving 3 pregnant heifers in the control group and 1 pregnant heifer in the vaccinated

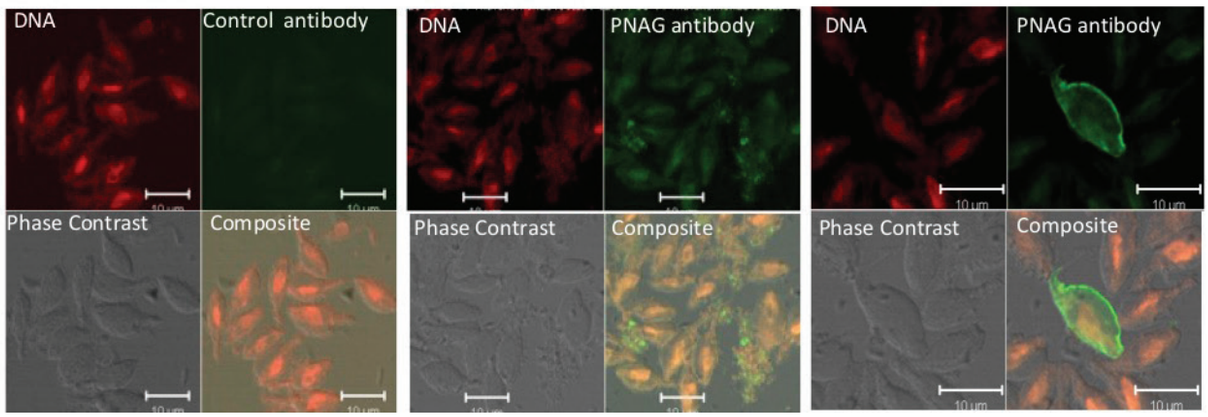

Figure 1. PNAG expression by *T. foetus*. Confocal microscopic images of control antibody treated (left) and anti-PNAG (green color on cells) treated cultures of *T. foetus* (center and right) showing PNAG expression both intracellular and on the surface.

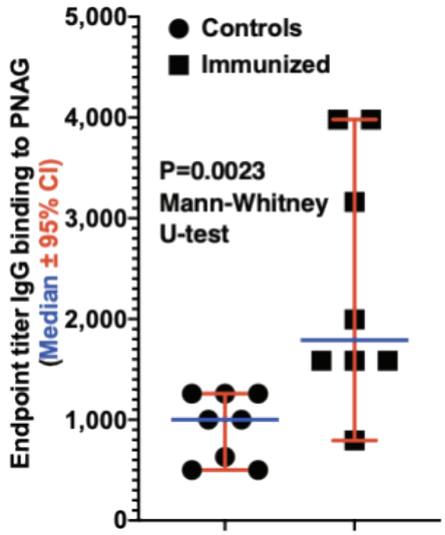

Figure 2. PNAG vaccination results in native-PNAG specific antibody titers. PNAG titers elicited with 200 ug PNAG vaccine for experimental challenge study.

group at the conclusion of the study. The rates of pregnancy were not significantly different between groups (P > 0.1).

In this study, 5/16 (31%) heifers were diagnosed as pregnant at d 32 post-insemination. This is a rate of pregnancy generally considered lower than most published studies utilizing estrus synchronization and timed insemination. Presumably, the pregnancy rate was reduced by the experimental infection. The rate may also have been lowered by the estrus synchronization and timed AI methods. The study used multiple doses of dinoprost[l] rather than a more intensive regimen in order to avoid altering the vaginal microbial flora by using an intravaginal hormone-releasing device. The study also avoided the use of progesterone implants or injections to minimize any vaginal or uterine effects from exogenous hormones.

Due to the lack of *in vivo* protection against *T. foetus* in immunized cows, we performed additional functional assays to determine a possible cause.

PNAG vaccine-induced antibodies mediated complement component C1q deposition but not bactericidal or opsonophagocytic killing of A. baumannii or S. aureus, respectively

Testing of the functional activity of the antibodies induced in the vaccinated cow's sera 35 d post-immunization demonstrated the PNAG-specific antibodies could fix bovine complement component C1q (Figure 3). Natural antibody to PNAG in sera of non-vaccinated, control cows did not deposit C1q onto the PNAG antigen, consistent with prior findings that natural antibodies are immunologically inert in these assays.[1,2,5] Surprisingly, sera obtained on d 35 from vaccinated cows did not mediate either bactericidal (Figure 3) or opsonic killing (data not shown) of target bacteria, demonstrating a lack of functional activity of these cow antibodies to PNAG.

Impact of adjuvant choice on cow antibody functional activity

Upon review of the literature it was suggested that cows preferentially require IgG2 antibodies for functional opsonic activity, which may be dependent on adjuvant type. The majority of antibody made to PNAG adjuvanted with Specol in the immunized cows was IgG1, not IgG2 (data not

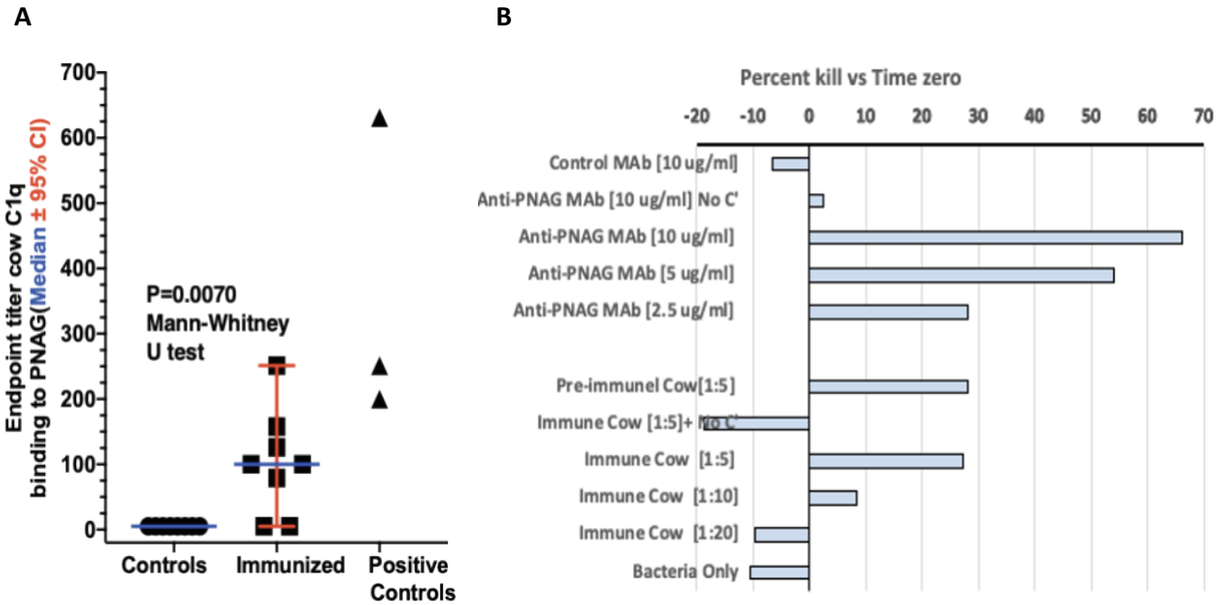

Figure 3. Functional activity of cow PNAG antibodies. (A) C1q deposition indicating low levels of C1q deposition by immune sera; (B) SBA titers confirming lack of function bactericidal activity against target bacterial strain.

shown), suggesting the isotype of IgG elicited by this vaccine and adjuvant combination may play a role in lack of antibody protective activity.

Conclusions

The fact that the adjuvant choice and resultant antibody may be impacting the functional activity of the antibodies is intriguing and will hopefully be addressed in the future. That notwithstanding, the study illustrates considerable potential for the development of a novel vaccine for *T. foetus*. First, unlike bulls, cows do clear the infection and, presumably, the immune system plays an important role. With the experimental challenge used in this study, only 1/16 cows appeared to clear the (vaginal) infection before embryonic signaling (approximately d 18) and before the beginning of placental attachment (approximately d 32). The challenge dose of 10^6 organisms may be a factor, and further studies should include a vaccination trial using natural insemination by infected bulls.

The current study also showed that vaginal infection is often tolerated during pregnancy without causing abortion.

In contrast to immunizing pigs and horses with a PNAG vaccine and Specol as adjuvant, that elicited functional and protective antibodies, in cows this same formulation elicited non-protective and non-functional antibodies as demonstrated by our *T. foetus* data.

While the current study did not protect against *T. foetus*-related early embryonic deaths, we suspect that the issue is not the lack of PNAG recognition by the antibody but rather, like in natural infections, the antibodies derived from immunization with Specol as an adjuvant elicited non-protective antibodies. Further work is therefore needed in this area for future development of an effective vaccine.

Endnotes

[a] Stimune® Immunogenic Adjuvant, Prionics, Lelystad, Netherlands, now part of Thermo Fischer Scientific, Waltham, MA
[b] Alopexx Enterprises, LLC, Concord, MA
[c] Thermo Fisher Scientific, Waltham, MA
[d] Sigma-Aldrich, St. Louis, MO
[e] AbD Serotec, Raleigh, NC
[f] Innovative Research, Novi, MI
[g] Cedarlane, Burlington, NC
[h] Stem Cell Technologies Inc., Cambridge, MA
[i] Boston Bioproducts, Ashland, MA
[j] Lutalyse, Zoetis, Parsippany, NJ
[k] Factrel, Zoetis, Parsippany, NJ
[l] In-Pouch TF Bovine, Biomed Diagnostics, White City, OR

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