Fractionated *Clostridium Septicum* Antigens for Turkey Clostridial Dermatitis Vaccines

D.N. Foster[1], C.A. Lancto[2] and L.K. Foster[1]

Departments of Animal Science[1] and Veterinary Biomedical Sciences[2]

University of Minnesota

St. Paul, MN 55108

Introduction

Gangrenous dermatitis, a.k.a. cellulitis, has increased in prevalence and severity in turkey populations over the last several years (Clark et al., 2010). Cellulitis has been associated with *S. aureus* and *E. coli,* and more recently found in field situations with Clostridial species, specifically *Clostridium septicum* (Tellez et al., 2009; Clark et al., 2010). *Clostridium septicum* is a large, gram-positive bacterial rod that grows anaerobically, forms spores and produces a lethal and necrotizing α-toxin that damages soft tissues due to its cytolytic membrane insertion and pore formation functions. We have now produced a recombinant protein that encodes the complete *Clostridium septicum* α-toxin with the exception of the cytolytic 28 amino acid domain in the middle of the polypeptide. When assayed for cytotoxicity this peptide did not lyse cells even at 1mg/ml purified protein whereas wild type α-toxin lysed cells at 10-50pg/ml. The protein, produced alone and in conjunction with a fusion peptide to increase stability was tested in a vaccination trial to determine if birds can mount a protective immune response when challenged with infectious *Clostridium septicum.*

Materials and Methods

Recombinant non-cytolytic α *toxin (NCAT) protein of* Clostridium septicum *expression and purification*. The 390 amino acid *Clostridium septicum* α toxin protein minus 28 internal amino acids (residues 204 to 231; Genbank # EA071361, Tweten and Melton) that comprised the cytolytic pore forming domain of the molecule (and deleted the major virulence factor allowing for safe handling of the recombinant protein) was synthesized as a 1190 base pair fragment by GeneArt AG (Regensburg, Germany). The non-cytolytic α toxin (NCAT) fragment contained multiple restriction sites at both 5' and 3' ends and was cloned into the pET32a expression vector (Novagen-EMD, Gibbstpwn, NJ) followed by transfection into *E. coli* Rosetta cells (Novagen-EMD) for protein expression. The NCAT protein was expressed with a 109 amino acid thioredoxin fusion partner (NCAT-TXN), which provides greater immunogenicity and stability to the recombinant α toxin protein. The His-tagged fusion peptide was expressed and purified after induction of the *E. coli* bacteria for 90 min with 0.1mM IPTG. In addition, in association with Nature Technology Corp. Lincoln, NE, the NCAT fragment was expressed alone (NCAT) or in conjuction with a maltose binding fusion protein ((NCAT-MBP), in two additional constructs. Due to greater protein expression efficiency, these constructs were selected to provide the protein used in the vaccine formulation.

Cytotoxicity assay. To verify that the recombinant *Clostridium septicum* α toxin protein (NCAT) was safe to use (i.e., non-cytolytic), a cytotoxic assay was performed essentially as described by Melton et al., (2004) using a T-lymphoblastoid cell line (Sup T1). The cytolytic levels of the wild type *Clostridium septicum* and mitomycin c (positive controls) were compared with the NCAT and NCAT-MBP peptides. Saline and culture media were used as negative controls.

Sup T1 cells were plated in culture media at 5 x 10^5 cells/ well in sterile 96 well reaction plates. All reactions were performed in triplicate. The growth media containing wild-type α-toxin was serially diluted by 5-fold increments and added to Sup T1 wells at dilutions ranging from neat to 78,125. Purified NCAT and NCAT-MBP proteins were added to wells at final concentrations of 100 µg, 50 µg, 25 µg, 10 µg and 2 µg. Following a19 hour incubation at 37˚C, 5% CO_2, ten microliters of CCK-8 dye (Dojindo Molecular Technologies, Rockville, MD) was added to each well. The plate was returned to the incubator for an additional two hours then centrifuged at 250 x *g* for 5 minutes. 50% cell death (tissue culture lethal dose, TCLD 50%) was determined by measuring absorbance at A_{450}.

Vaccine formulation. The Alhydrogel [$Al(OH_3)$] and AdjuPhos [$Al(PO_3)$] adjuvants were obtained commercially (Accurate Chemical Co., Westbury, NY). Vaccines were formulated at 50 µg protein and 0.3 mg of aluminum per 0.2ml dose. Protein and adjuvant were combined with 0.3% NaCl, pH 6.5 and mixed with rotation for 16 hours at 4˚C to absorb protein onto the aluminum salt by ionic attraction. Following absorption, free protein could not be detected in the supernatant of the suspension indicating that all of the protein had bound to the aluminum salt.

Serum Antibody ELISA. Blood samples were collected from each group of turkeys periodically during the vaccination trial and stored as serum at -20˚C. To determine the level of antibody specific to the recombinant NCAT protein, an ELISA assay was developed. Antigens were diluted in 0.2M carbonate-sodium bicarbonate buffer, pH 9.5 and allowed to bind to assay plates (Corning Life Sciences, Lowell, MA) in a total volume of 100 µl overnight at 4˚C. NCAT was plated at 10 µg/ml. Chicken IgY (Acris Antibodies, San Diego, CA) was plated at 1000, 500, 100, 50, 10, 5, and 1 ng/ml in dupicate wells to form a standard curve. Non-specific binding sites were blocked and 100 µl of turkey serum diluted 1:500 was added to wells and incubated overnight at 4˚C. The secondary antibody, 100 µl of rabbit anti-chicken/turkey IgG (Invitrogen, Carlsbad, CA), was added to wells at a 1:3000 dilution following five washings using TBS-T. Following incubation, the Amplex® UltraRed substrate (Invitrogen) was prepared following manufacture's instructions and added at100ul per well. Plates were incubated 15 minutes in the dark before 20 µl of stop solution (Invitrogen) prepared using the manufacturer's directions was added to all wells. Plates were read on a Fluoroscence plate reader with excitation at 544nm and emission at 590nm. Wells incubated with the secondary reagent were used as the blank reference value. Linear regression of the standard curve values was used to interpolate the levels of antibody in the turkey serum. These values were graphed and data was analyzed for significance by one-way ANOVA (Prism software, Graphpad).

Results and Discussion

We have used the pET expression vector system to produce recombinant *Clostridium septicum* α toxin protein with a fusion peptide (NCAT-TXN). Figure 1 shows SDS-Polyacrylamide gel electrophoresis (PAGE) using a 10-20% gradient

under denaturing conditions to express an approximate 61kDa recombinant thioredoxin/ α toxin fusion protein (NCAT-TXN). The peptides will be used for side-by-side comparison to the previously prepared NCAT and NCAT-MBP (Nature Technology Corp. Lincoln, NE).

We have used both the NCAT and NCAT-MBP for a cytotoxicity assay to be sure that deleting the internal 28 amino acids from the wild type α toxin protein rendered the peptide non-cytolytic and thus safe to use in vaccination trials. Figure 2 shows the results of adding increasing amounts of NCAT protein to the SupT lymphoid cells. The NCAT protein alone was *not* toxic to the SupT cells at concentrations of 100 µg/well (1 mg/ml). The NCAT-MBP showed increasing cytolytic effects at greater than 25 µg/well (250 µg/ml). For our vaccination trials, we are only using 50 µg/dosage which is well below the limits of cytotoxicity. The wild type *Clostridium septicum* causes cell lysis at levels between 10 and 50 pg/ml (data not shown).

A vaccination trial was performed using 40 day-old poults for each of the following vaccines: 1) 50 µg NCAT protein alone + Adjuvant; 2) 50 µg NCAT-MBP + Adjuvant; 3) 50 µg of the NCAT bacterin lysate used to prepare the purified NCAT protein + Adjuvant; and 4) 50 µg of the NCAT bacterin lysate used to prepare the purified NCAT protein without adjuvant. Vaccines were administered on days 2 and 21 (formulated with aluminum hydroxide adjuvant for groups 1 and 3 or with aluminum phosphate adjuvant for group 2). In addition, 30 poults were sham injected with either alum adjuvant or saline (control birds), and 10 poults were sacrificed at day 1 for analysis of pre-immune serum levels. Half of all birds were bled at 14, 21, 35 and 53d for ELISA. All birds were challenged at 53d with 0.5ml *Clostridium septicum* (1×10^7 cfu), monitored for mortality for 72h, then sacrificed by cervical dislocation with about 25% of the birds selected for necropsy. An additional experiment to test the effects of temperature and humidity on the mortality of the birds after being challenged was conducted by placing half of each group of vaccinated and controls birds at 64^oF to 72^oF with ambient humidity and the other half at 72^oF to 80^oF with approximately 10-15% increased humidity.. Table 1 shows the number and percentage of birds surviving challenge with the four vaccine formulations and the two control inoculations. The NCAT alone (blue tag) protected better than the NCAT-MBP (red tag) and the NCAT Bacterin +/- adjuvant (yellow and orange tags, respectively) provided 60-65% protection. This suggests that a formulation of bacterin alone with no adjuvant could be a cost effective vaccine. Of interest, 61% of all the birds that died following challenge were from the groups that had received the higher temperature and humidity. ELISA was performed on the sera samples collected throughout the trial. Figure 3 showed the following: 1) that the pre-immune sera reacting to NCAT was very low; 2) the NCAT alone (blue tag) showed significant titers at 21d but titers were not significant at 25 or 53d. This also correlates with the 67.5% survival rate. 3) the NCAT-MBP (red tag) did not show appreciable titers at 14, 21, 35 and 53d and this correlates with the 50% survival rates. It is possible that the immune response observed was primarily against the MBP and not the NCAT. Upon receiving the 2nd injection, the antibody titers should have increased 3 to 5 fold, or even greater. We did not

observe this dramatic increase. We theorize that the circulating antibody levels present in the birds was too high when the birds received the 2nd vaccination at 21d suggesting that subsequent immune responses were greatly reduced by clearance and that the 2nd vaccination should be delayed until around 35d; 4). The NCAT bacterin lysate + adjuvant (yellow tag) showed the highest titers at week 3 and was significant but again declined and became non-significant at 35 and 53d. The higher titers correlate with the 60% survival. Once again the reduced titers after 21d were probably due to antibody clearance; and 5) the NCAT bacterin alone (orange tag) did not show significant titers at any time tested, probably due to clearance, although the ELISA data did not correlate to the 65% survival rate.

Acknowledgements

We thank Dr. Michelle Kromm and the staff of the Jennie-O Turkey Store, Willmar, MN for their invaluable assistance in the vaccination trial. This work was supported by the Minnesota Turkey Research & Promotion Council and the Midwest Poultry Consortium/USDA Grant #2010-04.

References

Clark, S., R. Porter, B. McComb, R. Lippert, S. Olson, S. Nohner, and H. L. Shivaprasad. 2010. Clostridial Dermatitis and Cellulitis: An Emerging Disease of Turkeys. Avian Diseases 54:788-794

Melton, J.A., M.W. Parker, J. Rossjohn, J.T. Buckley, and R. K. Tweten. 2004. The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. J. Biol. Chem 279:14315-14322.

Tellez G. N. R. Pumford, M.J. Morgan, A.D. Wolfenden, and B. M. Hargis. 2009. Evidence for Clostridium septicum as a Primary Cause of Cellulitis in Commercial Turkeys. J. Vet Diagn Invest 21:374-377.

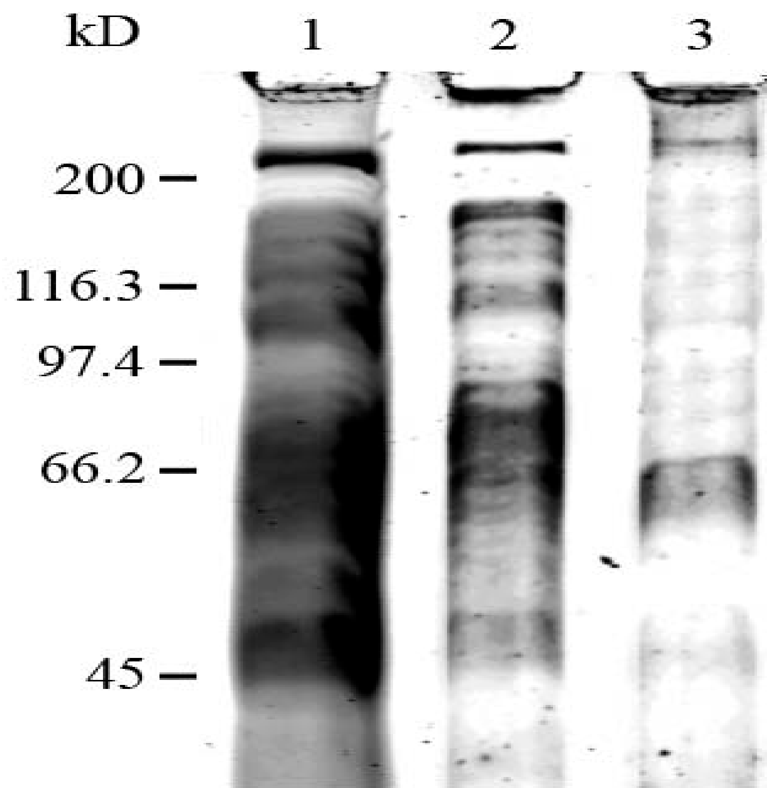

Figure 1. Gradient SDS-PAGE of recombinant α toxin / thioredoxin fusion protein (NCAT-TXN) using the pET32a expression vector. Lane 1, Total protein; Lane 2, Wash; and Lane 3, Fraction 1 showing the approximate 61kDa recombinant fusion protein (NCAT-TXN).

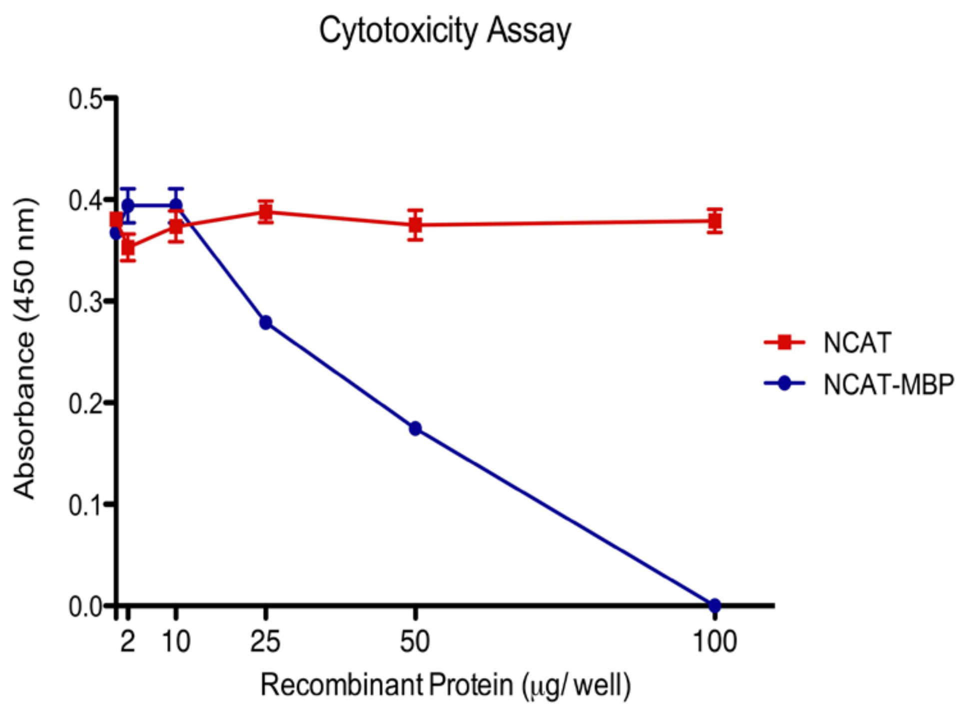

Figure 2. Cytotoxic assay fluorescence values at increasing protein concentrations for NCAT and NCAT-MBP recombinant proteins.

Table 1.

Vaccine	Tag identifier	# birds surviving challenge	% birds surviving challenge
NCAT + Adjuvant	Blue	27/40	67.5
NCAT-MBP+ Adjuvant	Red	20/40	50
NCAT Bacterin + Adjuvant	Yellow	24/40	60
NCAT Bacterin No Adjuvant	Orange	26/40	65
Negative control: Alum adjuvant	Brown	7/23	30
Negative control: NaCl	Pink	4/9	44

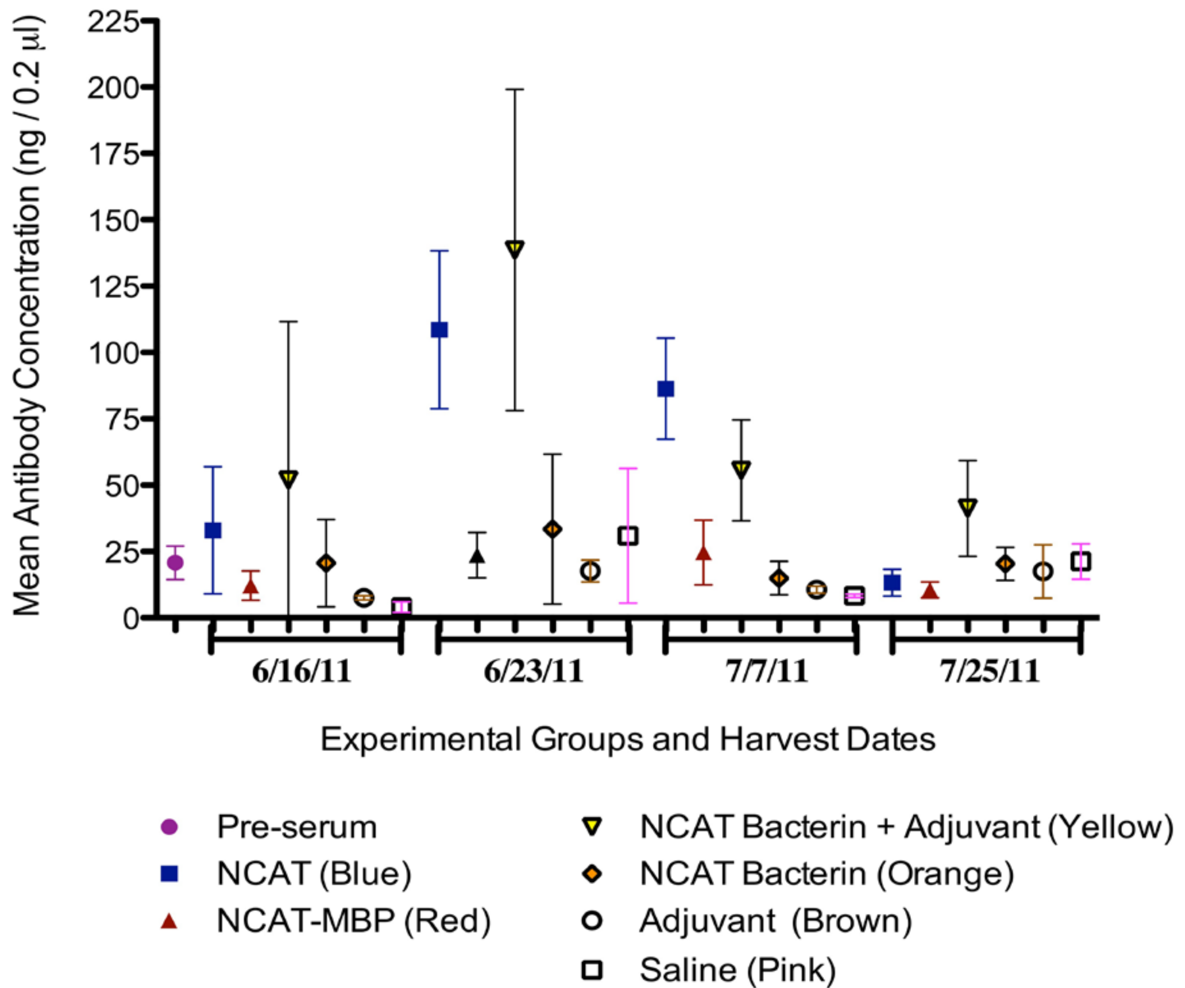

Figure 3. Levels of antibody specific to NCAT in turkey serum collected during the vaccination trial. Serum antibody was determined by ELISA and analyzed by one-way ANOVA. Data represents the mean and standard deviation of each experimental group.