Development of Immunogens to Protect Against Turkey Cellulitis, Part II

D.N. Foster[1], C.A. Lancto[2], R. Gangl[1], J. A. Williamson[3],J. M. Luke[3]

Departments of Animal Science[1] and Veterinary Biomedical Sciences[2]
University of Minnesota, St. Paul, MN 55108,
Nature Technology Corporation, Lincoln, NE 68521[3]

Introduction

Turkey cellulitis, aka, gangrenous dermatitis is a bacterial disease of turkeys that affects the skin and subcutaneous tissues and is caused by Clostridium species (usually *Clostridium septicum* and *perfringens)*. In recent years clostridial diseases in commercial poultry has been on the increase in commercial flocks (Ritter, G.D., 2009). Epidemiological evidence (Oliveira, 2009) suggests that *Clostridium septicum* is the causative agent in the majority of cellulitis outbreaks in Minnesota. Using recombinant molecular biology, we have produced seven subunit fragments of the α toxin polypeptide which is the lethal exotoxin secreted by *Clostridium septicum.* Each of the fragments were generated by PCR and then placed into three different expression vector systems that allows for the tagged recombinant peptide fragments to be isolated from non-tagged host bacterial proteins. The peptides corresponding to fragments of the *Clostridium septicum* α toxin polypeptide themselves are not toxic but when presented as a vaccine, should generate neutralizing antibodies and impart a protective immune response against *Clostridium septicum* toxin when the birds are challenged.

Materials and Methods

Three different expression systems were used to produce recombinant *Clostridium septicum* α toxin fusion peptide fragments. For the Qiagen vector system (Valencia, CA), single colonies of M15 [pREP4] bacteria harboring the cloned Clostridium septicum α toxin cDNA fragments 1 through 7 in the pQE40 expression vector were grown overnight at 37°C with shaking in 2xYT broth containing antibiotics, diluted 50 fold in fresh 2xYT media plus antibiotics and cultured until an $O.D._{600}$ of 0.6 was reached.

Protein induction was accomplished by the addition of 1mM IPTG and the bacteria were cultured for an additional 4-5 hours with vigorous shaking then pelleted and stored at -80˚C. Thawed bacteria pellets were weighed and resuspended in Native lysis buffer. containing 1X BugBuster™ (Novagen, EMD, Darmstadt, Germany). Recombinant lysozyme, benzonase, and a protease inhibitor cocktail were added per manufacturer instructions. Following incubation at room temperature for 30-45 minutes, the suspension was fractionated by centrifugation at 10,000 x *g* for 30 minutes and the resulting supernatant was removed as the native lysate. The insoluble pellet was washed twice with Native lysis buffer followed by resuspension in Denaturing lysis buffer by gentle rocking at room temperature for 15-20 minutes. Insoluble debris was removed by centrifugation at 10,000 x *g* for 30 minutes. All purifications were performed using Qiagen batch methods (both Native and Denaturing) as described by the manufacturer. Elution fractions of the recombinant DHFR (stabilizing peptide)/ α toxin fragment fusion products obtained from the column along with representative samples taken during the purification process were examined by SDS- Polyacrylamide gel electrophoresis (SDS-PAGE).

For the pET 32a+ vector system (Novagen) *Clostridium septicum* α toxin cDNA fragments 1 through 7 fused to DHFR were placed into the multiple cloning site of the vector downstream from the thioredoxin fusion peptide. The pET 32a+ recombinant vectors were transfected into Rosetta-gami competent bacteria that enhance protein folding (Novagen) and protein synthesis was induced by IPTG for 1-2h. The production and purification of recombinant thioredoxin/ α toxin fragment fusion products were essentially as describe above.

The last vector system used a proprietary process (Nature Technologies, Inc., U.S. Patent 2010/0125129 A1) to produce native (non-denatured) thermostable peptides as a flagellin/ α toxin fragment fusion product.

Results and Discussion

The Qiagen vector system allowed us to produce DHFR/ α toxin fragment peptide fusion products following induction of the host bacteria with IPTG. Figure 1 shows the results of protein fractionation using SDS-PAGE. There was a huge induction of the predicted 31kDa recombinant DHFR/ α toxin fragment fusion peptide (Lane 2; denatured lysate) compared to the uninduced bacteria control (Lane 1). Lanes 3 and 4 show purification of the 31kDa recombinant DHFR/ α toxin fragment fusion peptide from native and denatured lysate, respectively. While estimated levels of recombinant fusion protein were estimated to be in the 1-5 mg range from the denatured lysate, removal of the urea used to denature the bacterial inclusion bodies that harbored the recombinant fusion peptides caused the proteins to precipitate into misfolded insoluble material. We were able to recover 31kDa His-tagged recombinant peptides using Ni-agarose columns but purity (Lane 3) but recovery (Lanes 3 and 4) was disappointing.

To overcome recombinant proteins forming insoluble bacterial inclusion bodies, each of the α toxin – DHFR fusion protein fragments were cloned into the pET 32a+ vector system which allows recombinant peptides to be fused with a readily soluble thioredoxin protein that deposits the fusion peptides into the bacterial cytosol for extraction using methods that maintain the protein in a native conformation. We included the DHFR motif in this fusion protein since it increases *in vivo* protein stability and displays low immunogenicity. Figure 2 shows SDS-PAGE analysis of uninduced bacteria (Lane 1),

the native lysate from 1mM IPTG 1 hr. induced bacteria with no added DTT (Lane 2), and the His tag purified fusion protein (thioredoxin/ DHFR α toxin fragment 5) at the predicted 43 kDa. Lane 4 is the native lysate from 1mM IPTG 1 hr. induced bacteria with 4mM added DTT (to modify the folding rate of the recombinant thioredoxin/DHFR α toxin fragment fusion peptide), and the purified His tagged protein at 43 kDa (Lane 5). Based on a protein standard curve we recovered 24 mg/L of purified fusion protein.

In collaboration with Nature Technologies, Inc., we have begun to test recombinant fusion proteins made using their proprietary process for production of thermostable fusion peptides in a native conformation. Figure 3 shows the His-tagged purified recombinant flagellin/α toxin fragments 2, 3, 4, 5 and 6 fusion peptides, respectively, migrating at approximately 59 kDa as analyzed by SDS-PAGE. We have enough native purified recombinant flagellin/α toxin and thioredoxin/ DHFR α toxin fragments (10-15mg each) for an in vivo analysis to determine the degree of protection against *Clostridium septicum* when the birds are challenged. In an initial vaccination trial, newly hatched turkey poults were vaccinated with either 10 or 50ug purified recombinant flagellin/ α toxin fragments /0.2ml, with or without a booster given three weeks later using Freund's complete adjuvant before being challenged with *Clostridium septicum.* Unvaccinated challenged birds served as infection controls. Analysis of anti-*Clostridium septicum* antibody production was determined by ELISA using purified recombinant α toxin fragments, or recombinant fusion proteins (flagellin) as antigens. Antibody specificity to *Clostridium septicum* α toxin was determined by comparing antibody titers against the α toxin fragments to titers generated against the fusion proteins. Functionality of the antibody response was determined by *in vitro* neutralization assays, and by histological

examination of tissues harvested from all experimental groups. While there were antibodies raised against both the purified recombinant α toxin fragments as well as the fusion protein, results were somewhat inconclusive since the amount of antigen and a second vaccination didn't appreciably change antibody titers. Interestingly, not all unvaccinated control birds died when challenged due to high levels of circulating maternal antibodies to the *Clostridium septicum* α toxin. Additional vaccination protocols are planned using our recombinant purified *Clostridium septicum* α toxin subunit peptides.

Acknowledgements

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References

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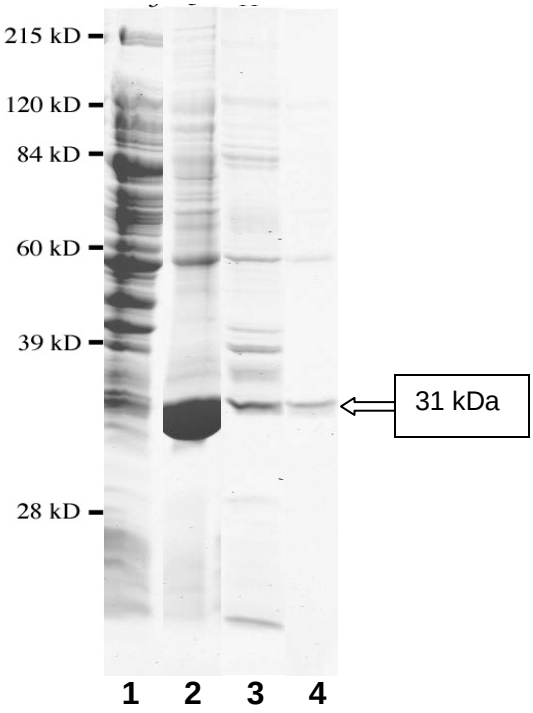

Figure 1. ***SDS-Polyacrylamide gel electrophoresis.*** Lane 1: Bacteria before induction; Lane 2: Lysate denatured in 8M urea; Lane 3: Native lysate recombinant DHFR / α toxin fragment peptide fusion product purified via 6x His-tag and Ni-agarose method; Lane 4: Denatured lysate recombinant DHFR / α toxin fragment peptide fusion product purified as in Lane 3 above.

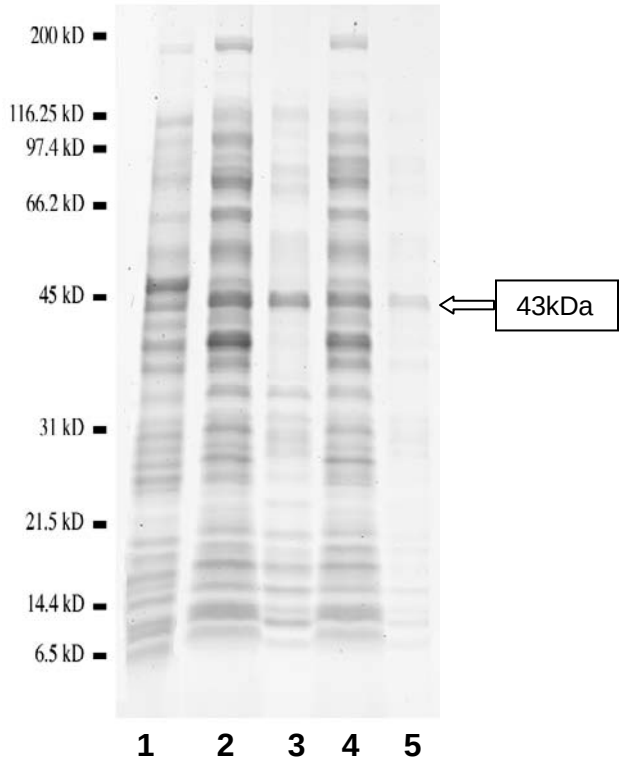

Figure 2. ***SDS-Polyacrylamide gel electrophoresis.*** Lane 1: Uninduced bacteria; Lane 2: Native lysate from 1mM IPTG 1hr induced bacteria with no DTT; Lane 3: Purified His tagged fusion protein (thioredoxin/ DHFR α toxin fragment 5); Lane 4: Native lysate from 1mM IPTG 1hr induced bacteria with 4mM DTT; Lane 5: Purified His tagged fusion protein (thioredoxin/DHFR α toxin fragment 5).

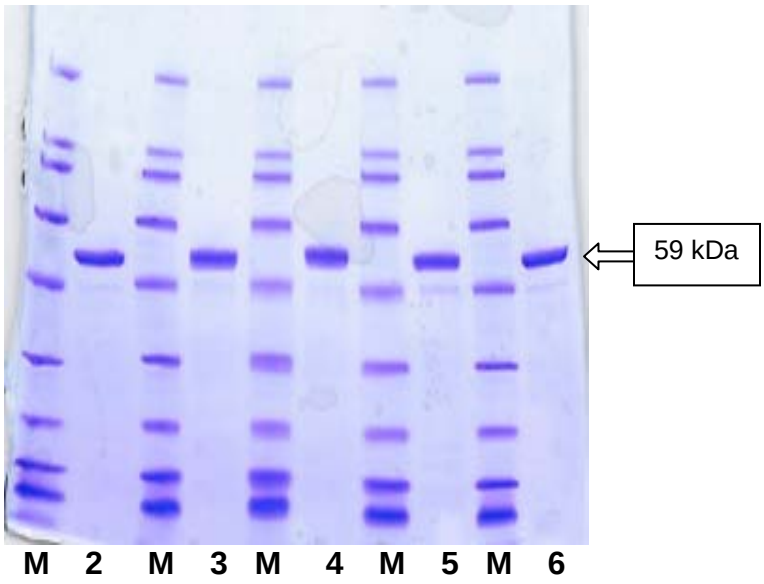

Figure 3. ***SDS-Polyacrylamide gel electrophoresis***. Recombinant fusion protein for Clostridium septicum α toxin fragments using Nature Technologies, Inc. proprietary process. M = Bio-Rad Broad Range marker; 2,3,4,5, and 6 correspond to α toxin fragments 2-6 as a 59kDa flagellin/α toxin fragment recombinant fusion peptide .