Title of Project: Breast blisters / cellulitis in turkeys: Genomic and proteomic studies on *Clostridium perfringens* type A strains.

PRINCIPAL INVESTIGATOR: Dr. Kakambi V. Nagaraja

CO-INVESTIGATORS: Dr. Srinand Sreevatsan
Dr. David A. Halvorson
Dr. Anil Thachil

NAME OF UNIVERSITY: University of Minnesota
DEPARTMENT: Veterinary and Biomedical Sciences

MAILING ADDRESS: 1971 Commonwealth Ave,
St. Paul, MN 55108

nagar001@tc.umn.edu
Ph: (612) 625-9704

DATE OF COMPLETION OF THE PROJECT: 01-01-2010

FUNDED BY U. S. POULTRY & EGG ASSOCIATION
INDUSTRY SUMMARY
Project # 649 Breast blisters/Cellulitis in Turkeys: Genomic and Proteomic studies on Clostridium perfringens Type A strains.

Breast blisters/Cellulitis has become serious concern for the turkey industry. This is accompanied by high mortality and carcass condemnation in the processing plants, resulting in heavy economic losses for turkey producers. Cellulitis in turkeys was named by the US Animal Health Association’s Transmissible Diseases of Poultry Committee as one of the top three industry concerns in 2008. Clostridium perfringens is one of the most frequently isolated organisms from lesions of breast blisters. Our preliminary studies at the University of Minnesota have demonstrated that turkeys vaccinated with a vaccine made from clinical isolates of virulent C. perfringens type A was found to be protective against clostridial cellulitis. It is believed that there are genetic differences in C. perfringens strains that may be associated with their phenotypic expression of virulence or their toxin production.

The objectives of this study included 1. To determine the genetic variations among C. perfringens isolates from cases of cellulitis in turkeys using Multi locus sequence typing (MLST). 2. To characterize the toxin profile of C perfringens and identifying immunogenic components recognized by turkey immune system to clostridial organisms using Difference Gel Electrophoresis (DiGE).

We characterized seven housekeeping genes and one toxin gene of C. perfringens isolates. The housekeeping genes examined were ddlA, dut, glpK, gmk, recA, sod, tpi and the toxin gene was plc. Among the eight loci examined plc locus had maximum number of alleles corroborating the fact that plc is highly diverse in nature genetically. The dN/dS ratio indicates that most of these polymorphisms found in the eight genes examined resulted in synonymous substitutions. We compared the level of sequence divergence by MLST from a dendrogram. Our studies indicated two major clusters among the C. perfringens isolates examined. The MLST results indicate that there is considerable genetic diversity among C. perfringens isolates from cellulitis cases unlike previous studies. C. perfringens from cellulitis cases showed no ancestral relationship with any of the reference strains like SM101, ATCC13124 and Strain13 whose genome sequences are published. Ongoing and future research work is being focused on establishing relationship between hemolysin toxin production and phylogenetic relatedness among these C. perfringens isolates.

Our SDS-PAGE analysis results demonstrated more than six reactive proteins of interest of C. perfringens. The major secretory toxins we identified from C. perfringens type A isolates cultured from breast blisters/Cellulitis cases by MALDI-TOF mass spectrometry were phospholipase, collagenase, hyaluronidase, Dnase, enolase, muramidase, pyruvate kinase and some hypothetical proteins. Hypothetical proteins were observed only in profiles of cellulitis producing C perfringens isolates whose role needs to be elucidated.

Our results suggest involvement of different toxins of C. perfringens that may play a role in pathogenesis and possibly in protective immune response against cellulitis in turkeys. Cellulitis inducing C. perfringens we examined differed in their secretory protein profile from non-cellulitis inducers but not in their genomic profiles. The results of this study enabled us to better understand the genotypic and phenotypic characteristics of C. perfringens type A involved in cellulitis in turkeys. This information of characterization of isolates from cases of breast Blisters would lead us to select a suitable vaccine candidate for the development of an effective protective vaccine against cellulitis in turkeys.
Final report on Project # 649 Breast blisters/Cellulitis in Turkeys: Genomic and Proteomic studies on *Clostridium perfringens* Type A strains.

**INTRODUCTION**

Breast blisters/Cellulitis has become serious concern for the turkey industry. This is accompanied by high mortality and carcass condemnation in the processing plants, resulting in heavy economic losses for turkey producers. *Clostridium perfringens* type A is one of the most frequently isolated organisms from lesions of breast blisters. This organism has been cultured from gangrenous dermatitis lesions in broiler chickens and is also identified as the organism responsible for breast blisters or cellulitis in turkeys. Thus it is worth noting that *Clostridium perfringens* type A is associated with distinct disease manifestations in birds.

During the Annual National Turkey Federation (NTF) Convention at Arizona in 2007, turkey cellulitis was discussed as an economic disease. The discussion was on current research available and knowledge gaps and research/needs and it was clear that there is a paucity of information on the risk factors and pathogenesis of cellulitis. The attendees felt the need for further understanding of cellulitis which is causing heavy economic loss for the turkey producers in US. Again in 2007, Turkey Veterinarians met during AAAP’s meeting in Washington DC at the NTF’s request and discussed the National situation of cellulitis in US. At the end of the meeting it was felt that the pathogenesis of the disease needs to be better understood in order to develop control strategies. Cellulitis in turkeys was named by the US Animal Health Association’s Transmissible Diseases of Poultry Committee as one of the top three industry concerns in 2008. To help address this request, the MTRPC conducted a Gold medal panel on Cellulitis in 2008 to further discuss this issue. The panel focused discussion on current research,
knowledge gaps and the research needs.

Our preliminary studies at the University of Minnesota demonstrated that turkeys vaccinated with a vaccine made from clinical isolates of virulent *C. perfringens* type A were found to be protective against clostridial cellulitis. It is speculated that there are genetic differences in *C. perfringens* strains and are associated with their phenotypic expression of virulence or toxin production. Currently, little is known about the genotype-phenotype relationship between *C. perfringens* type A isolates cultured from breast blisters/cellulitis cases. There were two objectives in this study. They were as follows.

The first objective of our study was to determine the genetic variations among *C. perfringens* isolates from cases of cellulitis in turkeys using Multi locus sequence typing (MLST).

The second objective of this study was to characterize the toxin profile of *C. perfringens* and identifying immunogenic components recognized by turkeys immune to clostridial cellulitis using Difference Gel Electrophoresis (DiGE) of culture supernatants of *C. perfringens* causing cellulitis.

**MATERIALS AND METHODS**

**Bacteria:**

*Clostridium perfringens* isolated from cases of cellulitis in turkeys from Minnesota were included in this study. Fifty isolates of *C. perfringens* were toxin typed and prepared for conducting partial sequencing of their genomic DNA. Briefly, after isolation of *C. perfringens* in cooked meat media, the pure colonies were grown in TSC with egg yolk and anaerobic sheep blood agar media. All the cultures were incubated anaerobically at 37°C for 48 hours using the
The pure cultures were identified using API 20 A kit, reverse CAMP test and toxin PCR.

**PCR conditions and sequencing:** Single isolated colony from the fresh culture was taken from one anaerobic blood agar plate into a microfuge tube containing 50µl of sterile water to extract DNA. The tubes with the culture were placed in water bath set at 100°C for 20 min and then kept immediately on ice. Later the tubes were centrifuged for 10 min at 15,000g and the supernatants were transferred to fresh tubes. DNA concentration was measured using a spectrophotometer. The DNA samples were diluted with water to adjust the DNA concentration to 50 ng/µl. PCR amplification of DNA was performed using 22U Taq DNA Polymerase (Platinum PCR Supermix, Invitrogen) in a reaction buffer containing 1.65mM MgCl₂, 0.22mM dNTPs and 1.5µM of each oligonucleotide primer. PCR was performed using 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. Final extension step was at 72°C for 5 mins. PCR products were purified with Qiagen PCR Purification Kit. Purified PCR products were submitted for sequencing along with forward and reverse primers to Biomedical Genomics Center, University of Minnesota.

**MLST (Multilocus sequence typing):** Genomic DNA was extracted using QIAquick PCR Purification Kit. The PCR reactions for the house keeping genes were standardized. The following locus of the DNA were sequenced namely plc (alpha toxin), dda (D-alanine-D-alanine ligase), dut (deoxyuridine triphosphatase), glpK (glycerol kinase), gmk (deoxyguanylate kinase), recA (recombinase), sod (superoxide dismutase) and tpi (triose phosphate isomerase). This was followed by phylogenetic analysis to determine the relationships between *C. perfringens*
isolates. The data was analyzed using Mega 4 software
(http://www.megasoftware.net/mega41.html). The sequence quality was read of all the Trace files in a chromatogram and poor quality sequences were trimmed. The sequence files were renamed according to the genes and isolate names. The sequences were then aligned using Mega 4 software and Polymorphisms were recorded. The sequences were realigned. Calculation of dN/dS ratios were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/ ) and SNAP (www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html). Sequence types were obtained using START.

Two dimensional gel electrophoresis (DiGE): The pure culture of C perfringens isolates were grown in fluid thioglycollate media initially. An 18 hour growth was transferred to DS media to enhance the toxin production under anaerobic incubation for 24 hours at 37°C. The total protein in the cultures was quantitated using a Pierce BCA protein Assay kit (Pierce Biotechnology). The samples were treated with protease inhibitors and aliquoted for SDS PAGE analysis. The proteins from the gel were then transferred to a PVDF membrane at 60v for 2 hours followed by reaction with primary antibodies. The sera used in this study were from turkeys which were vaccinated with C perfringens and found to be immune to cellulitis. HRP conjugated anti-Turkey IgY was used as secondary antibodies. Specific immunoreactive protein bands were visualized by chemiluminiscence imaging using an HRP luminol substrate kit (Bio-Rad Laboratories). A duplicate PVDF membrane from the sister gel was stained with Ponceau S to see the corresponding protein bands. We ran the same samples at more specific gel concentrations for each reactive protein depending on their molecular weight. Each reactive protein band was then identified by mass spectrometry.
MALDI-TOF mass spectrometry: The culture supernatants of selected ten isolates of C. perfringens isolates were treated with protease inhibitors and aliquoted for 2DiGE analysis. One hundred microgram of protein sample was subjected to Immobilized pH gradient (IPG) Isoelectric focusing (IEF) in the 1st Dimension. This separated the proteins by their charge (pI). Gel density in the SDS second dimension was fixed at 10%. The resulting gel was Coomassie Blue R-250 stained for viewing the protein spots. All gels were scanned using a GS-800 calibrated densitometer from which we obtained TIFF files of the image. We performed image analysis using Typhoon laser scanner and PDQuest software version 7.0 from Bio-Rad. A western blot was performed using convalescent sera. The reactive toxin components were spot picked and identified by MALDI-TOF mass spectrometry.

RESULTS AND DISCUSSION

Genetic variations among C. perfringens from cases of cellulitis in turkeys

Clostridium perfringens strains isolated from cellulitis cases were of toxino-type A by PCR. Seven housekeeping genes and one toxin gene were selected for characterization of C. perfringens isolates by MLST. The choice of gene for MLST was based on their use in MLST scheme for other bacterial species (1, 3). The housekeeping genes examined were ddlA, dut, glpK, gmk, recA, sod, tpi and the toxin gene plc. It is also been reported that only one copy of each of these housekeeping genes are present in the C. perfringens strain ATCC 13124 (2). Primers used for these MLST scheme were the same as reported before (4). Amplification and sequence analysis of the isolates examined were conducted using the protocol outlined before (4).
The average number of alleles per locus was found to be 4.3 with a minimum of 4 and maximum of 9. Among the eight loci examined plc locus had the maximum number of alleles (Table1) corroborating the fact that plc is highly diverse in nature genetically. The dN/dS ratio indicates that most of these polymorphisms found in the eight genes examined resulted in synonymous substitutions. Our findings are in agreement with previous reports [4]. This indicates that no functional change occurred in any of the final protein products.

To compare the level of sequence divergence as compared by MLST, a dendogram was constructed using Neighbor-joining method with bootstrap (500 replicates) showing genetic relatedness among the Clostridium isolates examined. Two major clusters were found among the C. perfringens isolates examined (Fig 1). Some of the isolates with less hemolytic titer fell into a class by itself. Besides that no such correlation between toxin production and genetic relatedness was observed. Members in both clusters varied considerably in their hemolytic toxin titer representing no direct connection between the expression of hemolytic toxin and phylogenetic relationship.

Multilocus allelic profile analysis generated sixteen different sequence types (ST). Mainly two clonal complexes were found and there were singletons too. Other studies reveal that C. perfringens from porcine origin and NE cases were clonal [4, 5]. However, there was no report regarding C. perfringens isolates from cellulitis cases in turkeys. The present study indicates that C. perfringens isolates from cellulitis cases do not show substantial clonality. We included sequence types from reference strains SM101, ATCC13124, Strain13 [6] in the dendrogram along with the C. perfringens isolates we examined to determine the phylogenetic relationship between them. All the reference strains fall in a subset of its own. This indicates that
*C. perfringens* from cellulitis cases showed no ancestral relationship with any of the reference strains.

In summary, MLST results indicate that there is considerable genetic diversity among *C. perfringens* isolates from cellulitis cases that we examined and the MLST scheme can be applied for typing of *C. perfringens* isolates causing cellulitis. Ongoing and future research work is focused on establishing a relationship between hemolysin toxin production and phylogenetic relatedness among the *C. perfringens* isolates.

**Characterization of toxin profiles of *C. perfringens* from cases of cellulitis in turkeys**

All the *C. perfringens* isolates examined were positive only for alpha toxin PCR indicating that they are all type A. This indicates that they express alpha toxin as the major toxin which has been reported before (7, 8).

SDS-PAGE analysis results demonstrated more than six reactive proteins of interest of *C. perfringens* on the PVDF membrane as luminiscent bands. *Clostridium perfringens* produces more than twenty different types of toxins and the toxin profile varies with the strain of bacteria (9, 10). *C. perfringens* produces extra cellular toxins including beta2 toxin, enterotoxin, perfringolysin, collagenase, lambda toxin, hyaluronodase, DNase, neuraminidase and urease (11).

Among toxins, alpha toxin was believed to be the predominant toxin responsible for *C. perfringens* pathogenicity as well as protection. But recent studies indicate that Alpha toxin does not have any role in the pathogenesis of necrotic enteritis caused by *C. perfringens* nor do they protect birds as demonstrated with an alpha toxin mutant of *C. perfringens* (12). Thompson et al.,
(2006) demonstrated that immunogens other than alpha-toxin are important in protective immunity against *C. perfringens* infection in broiler chickens.

Recently many hypothetical proteases and β2-toxins were found in virulent *C. perfringens* type A strains and that is believed to play a major role in eliciting a protective immune response against necrotic enteritis. A novel toxin NetB is now identified as a definitive virulence factor present in avian *C. perfringens* strains capable of causing necrotic enteritis in chickens (14). Another group of scientists recently demonstrated a hypothetical protein of 117 kDa suggestive of a protease found only in virulent *C. perfringens* type A causing necrotic enteritis. This protein is believed to play a major role in the development of necrotic enteritis and also in eliciting a protective immune response (14). It is also shown that the β2-toxin gene (*cpb2*) found in isolates of *C. perfringens* cultured from avian hosts are atypical compared to those found in pigs (4). It is not clear whether the above mentioned proteins play any role in the pathology of cellulitis in turkeys.

The major secretory toxins we identified in isolates from breast blisters/Cellulitis cases by MALDI-TOF mass spectrometry were phospholipase, collagenase, hyaluronidase, Dnase, enolase, muramidase, pyruvate kinase and hypothetical proteins. Hypothetical proteins were observed only in profiles of cellulitis producing *C perfringens* isolates. This needs to be further investigated.

Our results suggest involvement of different toxins of *C. perfringens* that may play a role in pathogenesis and protective immune response against cellulitis in turkeys. Cellulitis inducing *C. perfringens* we examined differs in their secretory protein profile from non-cellulitis inducers but not in their genomic profiles. The results of this study thus enabled us to better understand the genotypic and phenotypic characteristics of *C. perfringens* type A isolates involved in
cellulitis in turkeys.

References:


Table 1: Showing the number of alleles present in different gene loci of *C. perfringens*

<table>
<thead>
<tr>
<th>Gene Locus</th>
<th>Number of alleles</th>
<th>dN/dS</th>
</tr>
</thead>
<tbody>
<tr>
<td>plc</td>
<td>9</td>
<td>0.3526</td>
</tr>
<tr>
<td>ddlA</td>
<td>6</td>
<td>0.1072</td>
</tr>
<tr>
<td>dut</td>
<td>5</td>
<td>0.125</td>
</tr>
<tr>
<td>glpK</td>
<td>6</td>
<td>0.0004</td>
</tr>
<tr>
<td>gmk</td>
<td>5</td>
<td>0.0384</td>
</tr>
<tr>
<td>recA</td>
<td>5</td>
<td>0.478</td>
</tr>
<tr>
<td>sod</td>
<td>5</td>
<td>0.4981</td>
</tr>
<tr>
<td>tpi</td>
<td>4</td>
<td>0.0178</td>
</tr>
</tbody>
</table>

Figure 1. Dendrogram showing two distinct clusters of *C. perfringens* isolates.
Figure 2: Showing differences in the toxin profiles of a low potent *C. perfringens* isolate (left) and a high potent *C. perfringens* isolate (right).
LIST OF PRESENTATIONS/PUBLICATIONS


