

Title: A Vaccination Scheme For Establishing Passive Immunity To The  $\alpha$ -Toxin Produced By *Clostridium perfringens* In Neonatal Pigs

**Introduction:**

Necrotic enteritis is a severe disease of neonatal pigs caused mainly by *Clostridium perfringens* types A and C. The high morbidity rates between 30% and 50% and fatality rates as high as 100% are just cause to develop a vaccine, which would help to control this disease in the domestic pig population. The main cause of disease in neonatal pigs develops from production of several toxins by *C. perfringens*, particularly the  $\alpha$ -toxin produced by both types A and C. The difficulty preventing disease arises for several reasons. The route of transmission of the bacteria, which cause disease, is through the fecal oral route. Since this is the case, the neonatal piglets are exposed to the pathogen through suckling well before their own gut flora can be established to out-compete the pathogen. Without natural gut flora *C. perfringens* is able to prosper and cause disease. Additionally, the neonates' immune system is not able to adapt and mount an immune response to the pathogen.

Polymerase Chain Reactions (PCR) assays is used in clinics to test for the presence of *C. perfringens*. The toxin typing tests are usually performed using in vivo neutralization tests as well. *C. perfringens* typically become apparent when the spores germinate and vegetative cells multiply in tissues, which then lead to the bacteria spread among healthy cells and causing death (5).

**Ecology:**

*C. perfringens* is a widely widespread bacterium mostly due to its spore-forming ability (1). *C. perfringens* type A is distributed throughout North America as well as Europe (e.g. Belgium, Poland, Austria) (5). *C. perfringens* is broadly distributed in the environment and is also found in the intestinal tract of humans and animals. *C. perfringens* spores can be found on the human skin, soil and vagina (1). *C. perfringens* requires a low oxygen environment to stimulate spore germination, rapid vegetative growth and release of exotoxins (5). It is also important to note, that *C. perfringens* is a facultative anaerobe, enabling bacterial survival in varying conditions.

**Transmission/Clinical Features:**

*C. perfringens* spreads from exogenous source, but also from endogenous sources such as contamination of traumatic or surgical wounds or multiplication of organisms in the gut (6). Another type of contamination occurs when icy clostrum containing bacteria is ingested in large volumes (8). *C. perfringens* transmission can be divided into two forms. Anaerobic cellulite transmission occurs when the bacteria spread within the damaged necrotic muscle tissue produces toxin and gas. Most importantly, anaerobic cellulites are localized and do not spread to healthy muscles (1). The second form is called true mynecrosis and can lead to toxins produced in large muscles such as the thighs, buttocks and shoulders. The  $\alpha$  toxin diffuses into nearly all the healthy tissues, causing local necrosis and leading to continued clostridia growth and gas production (1). Another major way in which this bacteria spreads, would include the fecal-oral route, in which the bacteria recycles it back into the environment.

Type A,  $\alpha$  toxin of *C. perfringens* causes myonecrosis. Infected animals become depressed, anemic; have intense abdominal cramping and diarrhea, which occurs between 8-22 hours after consuming the bacteria (11). Other symptoms include tachycardia and blackened necrotic tissue, gangrenous infectious of uterus due to septic abortions and clostridial septicemia (1). The CPE etiological factor of pigs has shown that the spore count in affected animals is more than two logs higher than unaffected animals (8). This also suggests the *C. perfringens* is apart of the natural flora, but can become harmful, once the bacteria manifests the cells.

### **Animal Vaccine Proposal:**

Necrotic enteritis is a severe disease caused by *Clostridium perfringens*. The disease is quite common across several species including *Porcine*, *Equine*, *Ovine*, and *Bovine*. *C. perfringens* is able to cause disease by secreting several extracellular toxins such as  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\iota$  (5). These toxins are structurally defined as AB type toxins, this means that they contain two functional domains, which are bound by two disulfide bonds. The two domains have different functions; the A domain has a cell surface receptor binding function which induces target cells to phagocytose the toxin. Once this is completed the disulfide bonds are cleaved and the B portion of the toxin is able to cause cellular damage. It is known that in several cases *C. perfringens* is capable of producing multiple types of toxins to cause disease. B-toxin produced by the organism is usually responsible to the most severe cases of disease in *Ovine* and *Porcine* while  $\alpha$ -toxin is mostly responsible for disease in the *Equine* family. However,  $\alpha$ -toxin plays a key role in increasing the severity of disease in neonatal pigs 1-5 days old. We have chosen to target  $\alpha$ -toxin for vaccine development due to the lack of an effective vaccination for that toxin (5).

The most logical vaccine to develop is one, which gives the animal immunity to the toxin, which causes disease. This is why we have chosen to develop a vaccine, which would elicit immunity to the  $\alpha$ -toxin of both types A and C of *C. perfringens* by generating neutralizing antibodies. Based upon past work by Schoepe et al 2001, we believe that a  $\alpha$ -toxin variant produced by mutant strain 121A/91 would be an excellent antigen to introduce into the animal to induce the immune response. This particular  $\alpha$ -toxin variant is very important because it has been shown to have hemolytic function nor phospholipase activity, crucial functions of  $\alpha$ -toxin, which allows it to enter a cell and cause disease. Even more significant than the lack of enzymatic activity is the fact that this non-toxic form of the  $\alpha$ -toxin produced by this mutant is able to induce the production of antibodies that are able to neutralize  $\alpha$ -toxin produced by wild type strains of *C. perfringens* (7). Finally, since this particular toxoid has no pathogenic affects it could be given in a higher dose that if it were the wild type toxin without any modifications to the toxin.

As mentioned previously, neonatal piglets lack the ability to mount an immune response within the first few days after birth. Since this is the time frame when *C. perfringens* causes disease it would be pointless to immunize the piglets upon birth. The most logical course of vaccination would be through the sow before and during pregnancy. This is for a couple different reasons. First of all, the sow would be able to mount an immune response upon initial exposure and begin to develop an immune memory to the antigen. Upon the first booster vaccination the immune response would

be incited quicker and more efficiently. The sow would be vaccinated through two routes; injection and orally (7). There are two reasons for this. The vaccine delivered via injection would elicit a faster immune response and induce IgG production. The IgG produced by the mother would then be able to pass transplacentally to the fetuses. The oral route of vaccination would elicit IgA production due to the fact that this is the most prevalent type of antibody found in mucosal membranes such as those found in the gut. This is the most crucial part of our vaccination scheme due to the accumulation of IgA in the colostrums of the sow (5). Since the IgA would be passed directly into the gut of the neonates upon suckling after birth they would receive protection against wild type  $\alpha$ -toxin. This protection would be due to the neutralizing affect of the IgA against the wild type toxin. The IgA antibodies would be able to bind the region of the wild type toxin responsible for cell surface attachment and endocytosis (5).

Ultimately, the desired function of this vaccine is two fold. The first and primary function of the vaccine is to protect the neonates against  $\alpha$ -toxin and *C. perfringens* through the first few days after birth until their gut flora can be established. The second function is to illicit immunological memory in the sow in hopes to pass immunity on to neonates from future pregnancies without and vaccinations or with a single booster vaccination (7).

### **Innate Immune Response:**

Once the sow is orally inoculated with the  $\alpha$ -toxin variant isolated from *C. perfringens* 121A/91 the innate immune response will be activated to the foreign toxoid. The response will begin with the activation of macrophages in response to the toxoid's presence. The macrophage will engulf the toxoid and begin producing several cytokines. The cytokines, which will be produced, are IL-1, IL-6, IL8, and TNF- $\alpha$ . IL-1 will have the local effect of lymphocyte and vascular endothelium activation and will help to induce fever systemically. TNF- $\alpha$  will increase vascular permeability, attract other cells to the site, and aid in drainage to the lymph nodes. IL-6 and IL-8 will attract and activate lymphocytes and neutrophil (4). IL-6 has the additional affect of inducing acute phase protein production in the liver and also aids in the development of the inflammatory response by inducing fever. The activation of acute phase proteins and the presence of the interleukins will attract T-cells and TNF- $\alpha$  will attract dendritic cells, which are professional antigen presenting cells. Once these cells are present they are able to bind the toxoid. B-cells would also be involved at the site of the toxoid in order to bind antigen and return to the lymph nodes for activation by T-cells in the adaptive response. The binding of the antigen to the dendritic cell causes it to mature. The B, T, and dendritic cells will then migrate to the lymph nodes where the adaptive immune response will be initiated (4).

### **Adaptive immune response:**

Dendritic cells will migrate to the lymph nodes after they have taken up the  $\alpha$ -toxin variant introduced to the sow. Once in the lymph nodes the dendritic cells will be able to present the antigen on their MHC-II. At this time the dendritic cell will also be presenting another cell surface receptor CD-80. The expression of these two molecules along with the antigen will allow CD-4 T helper cells to attach to the dendritic cell via MHC-II and CD-80. CD-80 will join with CD-28 on the T-cell (4). Once the two cells

are joined the CD-4 T-cell signals will be passed between the two cells which cause the antigen presenting cell to express further surface proteins such as CD-86 and CD-40. CD-40 ligand will be produced on the surface of the T-cell due to the interaction of the CD-80 on the antigen presenting cell and CD-28 on the T-cell. CD-40 and CD-40 ligand will now bind, activating the CD4 T-cell. The activation of the CD-4 T-cell will induce the antigen presenting cell to express 41BBL, MHC-I, and B7. With the expression of these proteins the antigen presenting cell is able to activate CD-8 T-cells (4). This begins with the CD-8 T-cell expressing 41BB and MHC-I. These two proteins interact with 41BBL and MHC-I on the APC. The binding of 41BB to 41BBL fully activates the CD-8 T-cells. Now that the T-cells are active the CD-4 T-cell can activate B-cells which are presenting antigen on MHC-II which the CD-4 T-cell can recognize. Once attached via MHC-II and CD-40:CD40L the CD-4 T-cell can then release several cytokines, IL-4,5, and 6, which will signal the B-cell to differentiate and proliferate into antibody secreting plasma cells or memory cells. Once B-cells are stimulated to become antibody secreting plasma cell, they will begin producing and secreting antibodies, which are specific for the  $\alpha$ -toxin variant, introduced in vaccination. IgA is the type of antibody, which will be effective in neutralizing the toxoid. The reason IgA is used is because *C. perfringens* is an enteric pathogen, which produces its toxins in the gut of its hosts. IgA specific for the  $\alpha$ -toxin produced by *C. perfringens* will be able to neutralize the toxin by binding it in a way, which prevents the toxin from binding to epithelial cell surface receptors in the gut thus preventing damage (4).

#### **Vaccine Tests:**

Once the alpha homologue is isolated, we will be injecting a test group of pregnant sows. To determine the effectiveness of our vaccine we will use Flow Cytometry to measure cell immunity, as well as the ELISA test to measure humoral immunity. If these tests indicate that the vaccine is viable, then the neonatal and maternal pigs will be infected with a strain of *Clostridium perfringens* and the severity of their symptoms will be measured. In addition to these tests, four to eight hour old neonatal pigs will be challenged using wild type alpha toxin. The piglets challenged will be from both vaccinated and non-vaccinated sows, efficacy will be determined based on mortality rate. Necropsies will also be performed to determine exact cause of death (9).

#### **Flow Cytometry:**

Flow Cytometry will be used to measure the cellular immune response via the blood of the maternal sows, which will be centrifuged to remove erythrocytes. With this test, it will be expected that antigen specific cells will be at elevated levels as an effect of immune response. In order to utilize flow cytometry, it will be necessary to add flouochromes to the antibodies that are specific for antigen presenting cells. The intensity of the fluorescence is then recorded by the flow cytometer. This is done via a fluorescence activated cell sorter, which will separate the cells into groups by which cell specific antibody bound to them, if any. The following is a list of cell markers that will be targeted: CD 19 for B cells, CD4 for Th cells, CD8 for Tc cells, CD209 for dendritic cells, CD64 along with MHCII to distinguish macrophages and neutrophils. Along with this test a negative control will be run, this will be done by using spun down blood samples from maternal sows that have not been given the alpha toxin homologue (3).

### **ELISA (Enzyme Linked ImmunoSorbent Assay):**

The ELISA test will be our main test for focusing on humoral immunity, through the measuring of antibody production by B cells. Because our vaccine is designed to be both injected and given orally, both IgG and IgA will be measured.

IgG will be sampled from the blood of the Sows and the blood of the piglets, an ELISA test will be run on it against both the alpha homologue and against the actual alpha toxin. First, microtiter plates will be lined with either the alpha homologue or the actual alpha toxin. Second, blood samples that have been serially diluted will be introduced to the wells of the plates, in order to bind to the alpha homologue or alpha toxin. Next, a goat anti pig IgG will be introduced and allowed to bind the pig IgG. After that, a chromogenic indicator attached to additional anti pig Ig will be introduced, which a covalently linked enzyme in the Fc region of the goat IgG will bind (2). If the tests are positive the substrates will change color, to accurately measure this the plates will be run through a microplate reader and their absorbance values will be entered into a computer program. This data will then be used to determine how dilute the sample can be and still have enough antibody to elicit a positive (10).

IgA will be obtained from the colostrum of the sows, an ELISA test will be run on it against both the alpha toxin homologue and against the actual alpha toxin. First, microtiter plates will be lined with either the alpha homologue or the actual alpha toxin. Second, colostrum samples that have been serially diluted will be introduced to the wells of the plates, in order to bind to the alpha homologue or alpha toxin. Next, goat anti pig IgA will be introduced and allowed to bind the pig IgA. After that, a chromogenic indicator attached to additional anti pig Ig will be introduced, which a covalently linked enzyme in the Fc region of the goat IgA will bind (2). If the tests are positive, the substrates will change color; to accurately measure this, the plates will be run through a microplate reader and their absorbance values will be entered into a computer program. This data will then be used to determine how dilute the sample can be and still have enough antibodies to elicit a positive (10).

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