EFFECT OF CPG ON SWINE NEUTROPHILS AND USE AGAINST SALMONELLA IN PIGS

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Abstract Neutrophil oxidative burst/degranulation activities were measured using CpG in vitro stimulation. Neutrophils isolated from pigs 6 weeks of age were incubated with treatment for 1 hr at 39°C. Neutrophils had increased degranulation activity when treated with CpG when compared to the activity of cells treated with non CpG. Neutrophils treated with CpG exhibited an increase in oxidative burst. CpG resulted in levels of activity comparable to those observed with PMA stimulation. Two of the swine CpG (p2006 and pD19) were chosen for administration to swine to observe the effects on S. choleraesuis (SC) infection. Pigs were administered 1mg of CpG and subsequently challenged with SC. SC numbers in cecal contents in one CpG group were reduced by two to three logs when compared to control numbers and other CpG groups. In addition, reductions in SC positive rectal swabs, liver, spleen, jejunal, ileal, and colon colonization were observed.

Introduction The mammalian neutrophil is a first line of defense against invading pathogens and represents an important part of the innate immune defenses. The immunostimulatory activities of certain DNA antigens expressed by bacteria and other pathogens have been found to be linked to the recognition of these antigens by host receptors in the Toll-like receptor (TLR) family of receptors (Hemmi et al, 2000). Bacterial DNA has been found to be a potent immune stimulator and this activity has been linked to the presence of a high frequency of unmethylated CpG dinucleotides. CpG oligodeoxynucleotides (CpG-ODN) have been found to interact with TLR9 (Hemmi et al, 2000; Bauer et al, 2001). This interaction leads to a cascade of intracellular signaling resulting in gene expression and the production of immunomodulatory molecules including cytokines, helping to formulate aspects of both the innate and the adaptive host responses to invading organisms (Freytag, Clements 2005). CpG-ODN could be used as adjuvants for vaccines to increase their effectiveness as well as be used on their own as immune stimulators in domestic food animals and in human medicine (Freytag, Clements 2005; Verfaillie et al, 2005).

Salmonellae are known to colonize the gut of domestic swine and pose a risk for food born disease in humans. In addition, multiple serotypes are known to cause disease in swine including Salmonella choleraesuis, S. Typhimurium, and S. typhisuis, along with many others. Disease can vary from enterocolitis, septicemia, pneumonia to meningitis/encephalitis and mortality. In humans, food born disease from contaminated food products, including pork products, remains a continuing public health concern.

The purpose of the present investigation was to compare the effects of CpG-ODN known to stimulate immune responses in humans, pigs, and mice on neutrophils from swine in vitro and to determine if the oral administration of CpG-ODN in weaned pigs would reduce gut colonization and fecal excretion of S. choleraesuis in vivo.

Materials and Methods

Neutrophils were isolated from pigs (approx. 10kg body weight) using density gradient centrifugation. CpG-ODN The following CpG-ODN and non-CpG containing control (n2041) (Biosource International) were used for in vitro and in vivo (marked with “x”) assays:

- n2041 C*T*G*G*T*C*T*T*G*G*T*T*G*T*T*T*T*C*T*G*G
- CpG#17 G*T*C*G*T*G*T*C*G*T*C*G*T

Degranulation assay Neutrophils (8x106 cells/ml) were incubated with respective stimulant for 1 hour at 39°C. Tubes were then transferred to an ice bath for 10 minutes. Tubes were centrifuged at 250x g for 10min. at 4°C. Supernatants were then removed and stored at 4C. Each sample supernatant (25µl) or appropriate standard was added to each well in a costar flat bottom elisa plate #3915, non-treated, opaque and incubated with 50µl of freshly prepared substrate for 4h at 41°C in the dark. Stop solution was then added (200µl). Liberated 4-methylumbelliferone was
measured fluorimetrically (excitation: 355nm, emission: 460nm) with a GENios Plus Fluorescence Microplate Reader (Tecan US Inc.). Values were extrapolated from the standard curve.

**Oxidative burst assay** Production of ROS by swine neutrophils during oxidative burst was measured by the oxidation of DCFH-DA to fluorescent DCF. Neutrophils (1 ml at 8x10⁶ cells/ml) were incubated with stimulants and DCFH-DA (10µg/ml) for 1 h at room temperature. Aliquots (150µl) were then placed into black 96-well plates and the fluorescence was measured using a GENios Plus Fluorescence Microplate Reader (Tecan US Inc.) at 485 nm excitation and 530 nm emission wavelengths. The fluorescent units were recorded (RFU).

**In vivo S. choleraesuis study** Twelve pigs, average weight 26kg, were randomly assigned to 1 of 4 groups. Pigs were orally administered the respective treatment or control (1mg/pig) in 2ml volumes. Twenty four hours later, pigs were challenged by oral gavage with 109 colony-forming units (cfu) of S. choleraesuis. Rectal swabs were obtained daily from each pig and pigs in all groups were euthanized on day 7. At necropsy, tissues and contents from organs and the gut were cultured for the presence of S. choleraesuis using standard isolation techniques.

**Results** Results from *in vivo* culture of daily rectal swabs and tissue cultured for the presence of S. choleraesuis are presented below.

- CFU determinations in cecal contents were as follows:
  - 2041 – 3.4 x 10³ CFU/g content
  - p2006 – 3.5 x 10³ CFU/g content
  - pD19 – 10 CFU/g content
  - SC control – 4.4 x 10³ CFU/g content

Total # of positive samples/group in ICLN, Liver, spleen, jejunum, ileum, cecum, and colon combined:

- 2041 – 17/21
- p2006 – 15/21
- pD19 – 8/21
- SC control – 16/21

Total Number of daily rectal swabs positive for S. choleraesuis over 7 days:

- 2041 – 15/21
- p2006 – 17/21
- pD19 – 7/21
- SC control – 14/21

Results of the *in vitro* degranulation and oxidative burst assays are presented in Figures 1 and 2.

**Discussion** The use of CpG sequences as adjuvants or as direct immunostimulators presents a novel tool for the reduction of pathogens in food animals as well as people. The induction of the host’s endogenous immune mechanisms to fight infection and/or disease is also one way in which food animal producers could reduce the use of antibiotics as prophylactic or growth promoter measures, although a balance between growth potential of livestock and the energy costs of host immune functions must be considered.

In the present study, CpG DNA (pD19) was found to reduce *Salmonella* colonization in the gut and organs of swine. In addition, neutrophils isolated from the peripheral blood of pigs showed increased functional characteristics in the presence of CpG DNA (p2006, CpG 17, pD19) sequences than those not exposed to these sequences. Studies in chickens have also shown the effectiveness of CpG DNA in protecting against *Salmonella* infections and in stimulating the innate immune functions of the avian host (He et al, 2005). Although on their own, CpG...
sequences show promise in stimulating the host innate immune response, it is more likely that they can be used optimally as adjuvants for current or new vaccines against diseases and food-born pathogens.

**Conclusions** CpG DNA (pD19) was found to be effective in increasing the function of porcine neutrophils *in vitro*. *In vivo*, CpG DNA treatment of swine was found to reduce the shedding of *Salmonella* as well as reduce the colonization of lymph nodes, spleen, liver and the gastrointestinal tract of swine. Further study of the use of CpG sequences as adjuvants and as direct treatments aimed at the reduction of food born pathogens in swine need to be conducted.

**References**


