THE IMPORTANCE OF THE F4 RECEPTOR IN POST-WEANED PIGS IN ELICITING F4 SPECIFIC IMMUNE RESPONSES IN THE INTESTINE

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master's of Science

In the Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

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ABSTRACT

In this Master's dissertation, various doses of solubulized crude F4 fimbrial protein in conjunction with the adjuvants CpG ODN and porcine β-defensin 1 (pBD-1) were used to enhance the F4-specific intestinal immune response against Enterotoxigenic *Escherichia coli* (ETEC) F4 in post-weaned pigs. Using the mechanically shearing method we isolated the F4 fimbrial protein of ETEC with a molecular weight of 26 kDa. We verified this using a Western blot probed with a rabbit anti-F4 fimbrial antibody. Binding of the F4 fimbrial protein to the F4 receptor (F4R), present on the brush border of the villi in the small intestine of pigs, was demonstrated using an *in vitro* villus adhesion assay (IVVA). To demonstrate specificity rabbit polyclonal and mouse monoclonal anti-F4 antibodies, or the F4 protein were used to inhibit the adhesion of ETEC F4ac to F4R positive ($F4R^{pos}$) villi.

To examine immunogenicity of the 500 micrograms (μg) of the F4 were administered into surgically created jejunal gut-loops in pigs. Three weeks later Peyer's patches (PP) from immunized and control loops as well as gut-wall tissue were analyzed for their F4-specific antibody secreting cells (ASCs) by a modified enzyme linked immunosorbent spot (ELISPOT) assay. The F4-specific immune response in the serum was analyzed by an enzyme linked immunosorbant assay (ELISA). High numbers of F4-specific ASCs were isolated from the loops of pigs that contained high levels of the F4R. Conversely nominal or low numbers of F4-specific ASCs were found in loops of pigs expressing low levels of the F4R or no F4R ($F4R^{neg}$). The IVVA was used to categorize the pigs into either $F4R^{pos}$ or $F4R^{neg}$ animals.

Next three different concentrations of the crude F4 protein 50, 250, and 500 μg in the loops of individual pigs were used to analyze if dose affected the F4-specific immune response. Interestingly dose had no effect on the magnitude of the response. Therefore we hypothesized that the F4-specific immune response in the loops could be enhanced through the use of the adjuvants CpG ODN 2007 and pBD-1. The F4 protein was co-administered with either CpG ODN 2007 or pBD-1 and immune responses were assessed after 3 weeks. However neither CpG ODN 2007 nor PBD-1 at the doses used made an improvement in the immune response. Thus, these results demonstrated that the expression level of the F4R was the most important parameter for eliciting of the local immune response against the F4 protein. Furthermore our studies revealed that both $F4R^{neg}$ and $F4R^{pos}$ pigs responded to F4 immunization, however the former respond only nominally to F4-immunization in the loops. Moreover, an inverse relationship existed between the level of the F4-specific IgG in the serum and the F4-specific immune response seen in the loops. Thus our findings have important implications for oral vaccination using fimbrial based antigens (Ags) that utilize a receptor for their imunogenicity. Our results indicate that only animals with high levels of enterocyte F4R will have the ability to elicit high levels of protective F4-specific anti-fimbrial antibodies in their intestine after oral immunization. Therefore unless an effective adjuvant is available, animals with low to moderate levels of the fimbrial receptor in their small intestine will mount only weak immune responses making herd immunity after vaccination currently unattainable.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lorne Babiuk, who gave me the opportunity to pursue my graduate studies at the Vaccine & Infectious Disease Organization (VIDO) and guidance and constructive criticism during my graduate degree.

I would like to thank Dr. Volker Gerdts, who gave me the opportunity to work in his laboratory and for the guidance, discussions and valuable suggestions.

I would also like to thank members of my advisory committee, Drs. Lydden Polley, Philip Griebel, Volker Gerdts, Lorne Babiuk, Peter Bretscher, and Jim Xiang for their timely suggestions and help during my graduate program.

I would like to give a special thanks to Dr. Hugh Townsend for helping me with my statistical analysis.

I would also like to thank the lab members, Dr. Elahi Shrokollah, Julia Whale, Rachelle Buchanan, Janet Foley, and Dr. Monica Salles for their help throughout the various experiments.

I would also like to thank Joyce Sander (Human Resources Manager at VIDO), Administrative staff, GMP and animal care for their help. I would also like to thank Drs. Robert Brownlie, Kuldip Mirakhur, for sharing their expertise throughout my graduate program.

Lastly, I would like to thank my brother Peter for his timely support, and especially Darlene Penn for her love, kindness, and generosity throughout my entire graduate program.

Funding was provided by the Saskatchewan Agriculture Development Fund, the Canadian Institutes of Health Research, and the Canadian Network for Bacterial Pathogens of Swine

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ASC	Antibody-secreting cell
BCIP/NBT	5bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium
CCR6	Chemokine receptor 6
CpG ODN	Cytosine-Phosphate-Guanosine oligonucleotide
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISPOT	Enzyme-linked immunospot
F4 AG	F4 Antigen
F4R	F4 Receptor
GALT	Gut associated lymphoid tissue
HBD	Human beta defensin
hCAP	Human cathelicidin antimicrobial peptide
HIV	gp120 Human immunodeficiency virus-1 glycoprotein 120
iDC	Immature dendritic cell
IFN-γ	Interferon gamma
IFN-α	Interferon alpha
Ig	Immunoglobulin
IκB	Inhibitory kappa B Protein
IL	Interleukin
IMTGP	Intestinal mucin type glycoproteins
IPP	Ileal peyer's patches
IRAK	Interleukin -1 receptor associated kinase
IVVA	*In vitro* villous adhesion assay
JPP	Jejunal peyer's patches
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
NFκB	Nuclear Factor kappa B
NK	Natural killer cells
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
pBD-1	Porcine beta defensin-1
PBMC	Peripheral blood mononuclear cells
PBSA	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cells
PMSF	Phenylmethylsulphonylfluoride
SIg	Secretory immunoglobulin
TCR	T cell receptor
TIR	Toll interleukin-1 receptor
TLR9	Toll like receptor 9
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor associated factor 6
VIDO	Vaccine & Infectious Disease Organization

1. REVIEW OF LITERATURE

1.1 Diarrheagenic enterotoxigenic *Escherichia coli* (ETEC)

E. coli are motile Gram-negative bacteria that occupy the intestines of humans and animals as commensals or as pathogens. The genome consists of a singular circular DNA molecule of 4 x 10^6 base pairs with a molecular weight of ~ 2.5 x 10^9 Daltons. *E. coli* are serotyped based on their O lipopolysaccharide (LPS) and H (flagellar) surface antigen (Ag) profiles. Using a combination of O and H Ags there are over 10,000 possible O:H serotypes of *E.coli* [1] . The K or capsular Ags, also used for serotyping, were historically used to represent fimbrial Ags because of their intimate association with the capsule. However, a new nomenclature was introduced which includes classifying fimbriae as F Ags. Fimbriae are important virulence factors that allow bacteria such as ETEC to adhere to mucosal surfaces preventing their removal by normal intestinal processes such as peristalsis.

ETEC possess five different fimbrial subtypes, F4 (K88), F18, F5 (K99), F6 (987P), and F41 [2]. F5, F6, and F41 fimbriae are predominantly associated with ETEC that cause scours in neonatal pigs, whereas the F4 and F18 adhesins are found on ETEC that predominantly cause post-weaning colibacillosis (PWC)[3]. Once colonization is established, ETEC can elaborate heat-labile (LT), heat-stable (ST), and East-1 enterotoxins that act upon intestinal enterocytes causing secretory diarrhea. A variant of

ETEC known as ETEC/STEC (shiga toxin *E. coli*) can produce, in addition to LT and ST enterotoxins, Shiga-toxin that allows STEC to cause edema disease [4]. Another type of diarheagenic *E.coli* that occasionally cause PWC are enteropathogenic *E. coli* (EPEC). EPEC cause attaching and effacing lesions, with effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane resulting in diarrhea without elaborating toxins [4].

1.2 Enterotoxins of ETEC F4 responsible for post-weaning diarrhea in pigs

ETEC F4 infections are responsible for significant death and morbidity in neonatal and post-weaned pigs. ETEC expressing F4 fimbriae adhere to F4R on intestinal epithelial cells of F4R positive pigs ($F4R^{pos}$) only, resulting in colonization and subsequent enterotoxin secretion that leads to osmotic diarrhea in piglets [5]. Depending upon the strain, ETEC F4 express heat-labile (LT), heat-stable (ST), and or EAST 1 enterotoxins which cause diarrhea [3, 4]. LT comes in two variants namely LT1 and LT2. The former resembles cholera toxin in its protein sequence, receptor targeting, and enzymatic activity, LT2 is not associated with disease [4, 6]. LT1 is composed of 5 B subunits and one A subunit. The B subunit is responsible for binding to the receptor, whereas the A subunit contains enzymatic activity that stimulates production of cyclic adenosine monophosphate (cAMP). Increased levels of cAMP lead to a number of intracellular changes from cytoskeleton remodeling to increased activity in membrane transporters. These changes lead to a net secretion of chlorine and bicarbonate from intestinal cells, which eventually results in diarrhea [4, 6]. ST also comes in two variants, STa and STb, both causing diarrhea. STa induces increased levels of cyclic guanlyate

monophosphate (cGMP) which activates anion channels facilitating the passage of chlorine out of the cell and into the lumen and leading to osmotic diarrhea [5, 6]. STa is also responsible for initiating intracellular cytoskelatal rearrangement which contributes to the intestinal secretory response [5]. STb on the other hand is thought to stimulate increased production of serotonin and prostaglandins from intestinal cells resulting in increased levels of cAMP leading to diarrhea [7, 8]. Lastly, East-1 found in association with LT1 and STb shares considerable amino acid sequence homology with STa and acts through the second messenger, cGMP, to cause diarrhea [9].

1.3 The fimbriae of the *enterobacteriaciae*

Fimbriae are 0.5-1.5 microns long proteinaceous appendages peritrichously distributed at the surface of the bacterium that allow adherence of the bacteria to fimbriae-specific receptors [10]. Between 100-300 fimbriae are present per bacterium. Fimbriae are classified according to the presence and position of various amino acids (aa) in the primary sequence of their major fimbrial subunit [11]. The F4 fimbriae are placed into a class that do not contain the amino acid cysteine and contain a unique penultimate tyrosine at their carboxyl terminal end [11]. Fimbriae are transcribed from operons that can have either a plasmid or chromosomal location [11]. Transcription is either governed through regulatory molecules such as leucine responsive regulatory protein (Lrp) and catabolite activator protein (CAP), or is turned on or off through methylation-dependant phase variation via conserved DNA sequences known as GATC boxes [11-13]. Regardless of the type of regulation, a number of environmental factors such as carbon availability, pH, and temperature influence fimbrial biosynthesis [14]. Thus, fimbrial production in bacteria

is a multifactorial process involving global regulators, complex molecular mechanisms, and environmental conditions.

1.4 F4 fimbriae of ETEC

The F4 fimbriae are lectins that bind to glycoprotein receptors located on the villi lining the small intestine of pigs [15]. The genes faeA to faeJ are located on a plasmid and give rise to the corresponding proteins FaeA to FaeJ whose functions range from chaperones to building blocks for the F4 fimbriae [16]. The F4 has a molecular weight of approximately 26 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [17]. F4 fimbriae are ~ 1 um in length and are primarily composed of hundreds of repeating major subunits known as FaeG with a relatively small number of minor subunits interspersed throughout the structure. The minor subunits are not only part of the fimbrial structure but have important functions as well. For example, FaeC acts as a nidus for the addition of major subunits to begin fimbrial synthesis, FaeH and FaeF are interspersed throughout the fimbrial structure acting as scaffolding for the newly forming fimbriae [18]. Further, the minor fimbrial subunit FaeA acts as a repressor of fimbrial synthesis, the functions of FaeB, FaeI, and Fae J are unknown, FaeD acts as an outer membrane usher, and FaeE represents a periplasmic chaperone [12, 16, 19, 20]. The assembly of the F4 fimbriae begins with the expression of genes *fae*B through *fae*J, whose rate of transcription is influenced by the level of the repressor FaeA. FaeC initiates F4 production as it acts as the nidus to which copious amounts of FaeG subunits are added with the minor subunits FaeH and FaeF added at integral locations throughout the

structure to permit proper elongation. The completed fimbriae is anchored to the membrane by FaeD (Figure 1.1).

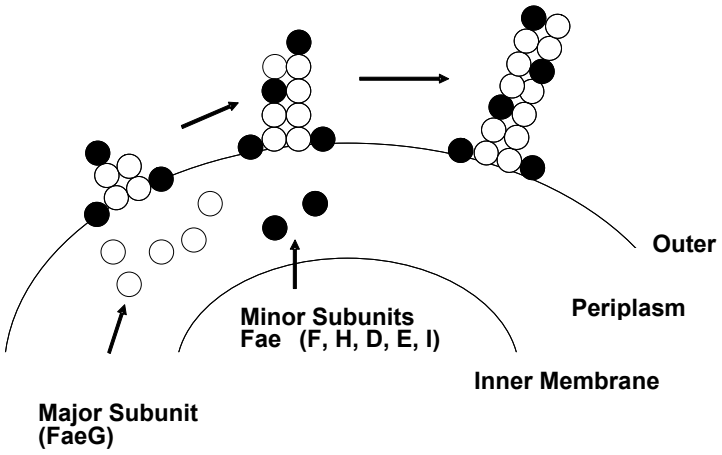


Figure 1.1 The expression of the major and minor subunits is followed by their incorporation into nascent fimbriae. The major subunit, FaeG, comprises the bulk of the fimbrial structure, with the minor subunits, FaeC and FaeF interspersed sparingly throughout. The minor subunit FaeE acts as a periplasmic chaperone with FaeD situated in the outer membrane acting as an anchor for the structure.

This complex biosynthesis can give rise to three fimbrial variants 'ab', 'ac', and 'ad' of varying lengths. For example, F4ab and F4ad are 264 aa long, whereas F4ac is 262 aa long [20]. The distinction between the fimbrial variants is based on differences in their primary aa sequence, which results in variants that all share common 'a' epitopes but contain unique 'b', 'c', or 'd' determinants [20, 21]. Aside from their unique antigenic differences, all three F4 variants have their receptor binding sites defined by interactions between their variable and conserved regions [18, 22]. Interestingly all F4 fimbrial variants bind the F4R with equal avidity.

1.5 F4R

The F4R is located in the villous brush border of susceptible pigs, where it facilitates binding of ETEC F4 to intestinal villi leading to colonization and diarrheal disease [23]. The enterocyte F4Rs are a group of glycoproteins that vary in size with molecular weights ranging from 45-240 kDa [24, 25]. These F4Rs have different specificities that allow them to bind one, two, or all of the ETEC F4 variants. For example the 210 and 240 kDa F4Rs bind F4ab and F4ac only, whereas F4Rs with molecular weights ranging from 45-70 kDa bind all three F4 variants [24, 25]. The F4R is inherited in a Mendelian fashion with the gene for F4R expression being a dominant trait [23]. Its gene has been mapped to the region q41 -- q44 on chromosome 13 [26].

A reliable genetic test to categorize pigs as $F4R^{pos}$ or $F4R^{neg}$ is not currently available. Thus, the *in-vitro* villous adhesion assay (IVVA), an *in-vitro* test in which bacteria adhering to pig intestinal villi are enumerated to detect the presence of the F4R, is currently the only method to classify pigs as F4R negative ($F4R^{neg}$) or $F4R^{pos}$ [27]. Receptor classification, however, is more complex than a simple positive or negative categorization. For example, when the F4 variants ab, ac, and ad, are considered within the context of their adherence patterns, six adhesive phenotypes exist: phenotype A (binds all three variants), phenotype B (binds F4ab and F4ac only), phenotype C (binds F4ab and F4ad only), phenotype D (binds F4ad only), phenotype E (no F4R, therefore no binding of any variant of ETEC F4) and phenotype F (binds F4ab only) [28]. This classification scheme enables us to determine which variants are endemic in a particular region of the world. For example, F4ad used to be commonly found in Europe, however today, F4ac is the most common type detected in Europe and throughout the world.

The enterocyte F4R is expressed in pigs throughout their entire life time. However, their expression decreases with age making older pigs less susceptible to disease [2, 29]. Interestingly the F4R is also found in pig intestinal mucus due to enterocyte turnover, their function is to bind ETEC F4 and aid in their removal from the intestine [30]. Therefore, F4R in the mucus function to protect the neonatal pig from infection with *E.coli*. Presence of F4R in the mucus ceases by 6 months and declines significantly at the time of weaning [24, 29]. In this study, we were solely concerned with the role of the enterocyte F4R in influencing the strength of the F4-specific immune response in pig gut-loops.

1.6 Clinical signs of PWC

ETEC F4 infection in post-weaned pigs can result in diarrhea that typically begins 16-36 hours post infection. Depending upon the severity of the diarrhea, feces can range in consistency from porridge like to liquid in severe diarrhea. Other clinical symptoms include fever, lethargy, discoloration of the ears and abdomen, with the tail and skin around the anus becoming wet and pasted with fecal material. Loss of appetite frequently occurs and, if prolonged, can result in retarded growth of the pig [2]. Post mortem analysis can reveal an abnormal accumulation of blood and fluid within the intestine [2].

A number of methods are used to diagnose ETEC F4 infection in post-weaned pigs including serotyping to identify the strain (i.e. O149, O8, O138, O141) based on Ags from the LPS, and unique epitopes found on different fimbriae (i.e. the major subunit is usually targeted) [31]. PCR and DNA probes are used to detect the presence of virulence

genes such as fimbriae and toxins [32, 33]. ELISA can also be used to serologically detect the presence of fimbriae or toxins [34].

ETEC expressing F18 is also frequently found as a causative agent in PWC [35, 36]. Therefore, some diagnostic techniques are required to distinguish between ETEC F4 and ETEC F18 strains as the causative agent for PWC. One method of differentiation is the use of PCR to detect *fedA* (the major pilus subunit gene) or *fae*G the adhesins involved in adherence in ETEC F18 and ETEC F4, respectively [20, 37]. The enterotoxins LT and ST are good markers to verify ETEC as the etiological agent in PWC because ETEC F4 typically secretes LT whereas ETEC F18 is more commonly associated with the toxins STa and STb [3, 38]. Immunological (ELISA and agglutination) or molecular (PCR, or DNA probes) methods could also be used to detect the presence of these proteins or genes, respectively [32-34]. Therefore, to identify the strain(s) of ETEC responsible for an outbreak of PWC in pigs, more than one assay may be required.

1.7 Vaccination against PWC

A commonly used method to prevent disease in livestock is vaccination. For example, maternal immunization represents a way to protect neonatal pigs against ETEC infection. Sows are typically immunized with a combination of fimbrial Ags commonly associated with neonatal diarrhea, and produce antifimbrial antibodies that are passively transferred via colostrum and milk to suckling piglets [39]. Unfortunately, this lactogenic immunity is no longer available to the post-weaned pig leaving it susceptible to ETEC infection. Thus, active immunization of the piglet is required to confer protection against PWC. Unfortunately, parenteral immunization of the piglet is not effective since

parenteral immunization fails to stimulate sufficient mucosal immune responses in the small intestine [40]. Therefore, oral vaccines are required to elicit an intestinal mucosal immune response that can confer protection against PWC.

At the present time no commercial vaccine exists to prevent PWC even though a number of F4 fimbrial-based vaccines have been tested in pigs. For example, live attenuated *E.coli* expressing F4 fimbriae administered to 4 week old pigs has been tested but did not elicit enough memory F4-specific T cells that could be protective against challenge infection [41]. However, if weaned pigs were co injected intramuscularly with levamisole, then orally vaccinated with live attenuated *E.coli* expressing F4 fimbriae some level of protection was achieved, thus adjuvant has the potential to enhance the immune response to the F4 in pigs [42]. Several studies, however, have investigated the utility of using purified F4 fimbriae instead of attenuated *E.coli* as a source of the F4 Ag. Van den Broeck et al. 1999 vaccinated weaned pigs orally with purified F4 fimbriae and found it effective in stimulating the mucosal immune response as well as conferring protection against challenge with ETEC F4. Furthermore, the same authors demonstrated that only $F4R^{pos}$ pigs responded to oral immunization with the F4 fimbriae [43]. However, the efficacy of oral immunization with F4 fimbriae leads to variable outcomes, as our group carried out a similar vaccination/challenge experiment and found no enhanced F4-specific immunity or protection against infection with ETEC F4 [44]. Nonetheless, the F4 fimbriae or its major subunit FaeG continue to be tested for use in vaccines in a variety of ways. For example, pure F4 has been co-injected parenterally with an active metabolite of vitamin D into weaned pigs. Protection however was minimal and no F4-specific IgA-ASC were found in the GALT of vaccinated pigs after immunization [45]. Oral

immunization with purified F4 coated in an enteric formulation designed to allow the F4 to reach the intestine in its most immunizing conformation is still being tested but F4 dissociation from the coating polymer is currently a problem making this method of delivery ineffective [46]. On the otherhand feeding diets containing egg yolk antibodies from laying hens hyper immunized with F4 fimbriae then incorporated into feed have been shown to reduce the severity of diarrhea and incidence of mortality due to ETEC F4 [47]. Other methods involving feed are currently being developed for example the incorporation of the FaeG subunit into plants such as alfalfa and transgenic tobacco, could eventually be used for cheap large-scale production of the F4 fimbrial protein [48, 49]. Currently, however, no method employing the F4 fimbriae either in its purified form or as a live vaccine is totally effective in preventing PWC in pigs.

1.8 Mucosal immune system development and function in pigs

The mucosal immune system in pigs develops within the first weeks of life. The development of the pigs SIg response in the lamina propria begins prenatally, unimpeded by passive maternal immunity, and continually develops until 3 months of age [50]. Thus, the weaned pig is capable of eliciting an adaptive immune response in the gut as jejunal and ileal PPs are functional having a immunoglobulin repertoire of SIg that could theoretically afford protection against ETEC F4 [50].

1.9 Induction of F4-specific immunity in the pig intestine

It is believed that once an Ag is delivered orally it will be taken up by specialized enterocytes known as M cells that overlay the PPs. M cells facilitate the transport of Ags

to dendritic cells (DCs) macrophages, and eventually to lymphocytes [51]. The presence of professional Ag presenting cells in the dome region of PPs allows for stimulation of T-helper cells, which provide specific help for IgA isotype production within the intestine [52]. Pigs have three different types of Peyer's patches, ilieal Peyer's patches (IPP), jejunal Peyer's patches (JPP), and spiral colon Peyer's patches [53]. The pig JPP and IPP are both secondary lymphoid organs with the former having a more pronounced effect on immune induction than the latter [46, 50]. Other tissues in the porcine gut can also function to induce mucosal immunity. For example, enterocytes take up Ag directly and pass it on to underlying APCs or present it via MHC I. However, presentation is limited because epithelial cells fail to express the co-stimulatory molecules needed to effectively present Ag [54]. Furthermore, studies in mice have shown that Ag within the intestine can also be taken up by DCs that survey the lumen via transepithelial dendrites, thus acting as sites of induction [55, 56].

1.10 Gut-loop model

Conventional studies involving oral immunization with soluble F4 Ag in weaned pigs have revealed that the F4R is required to elicit an F4-specific immune response in the intestine of $F4R^{pos}$ pigs [43]. Unfortunately, trying to use this method to understand the relationship between the expression level of the F4R and the local F4-specific immune response in the gut is impractical and very costly. This is because the pig is an outbred species that would necessitate the use of several pigs in order to generate cohorts with similar expression levels of the F4R. Moreover, the process of orally delivering soluble F4 Ag to several of these pigs in itself poses a problem because it requires physical

manipulation of the pig, injecting the inoculum, and ineffective delivery of the F4 Ag to the small intestine after being subject to gastric and intestinal enzymes. Therefore, a more effective method is required to analyze the local immune responses within the small intestine. In the past, investigators created Thiry-Vella loops to examine these types of interactions [57]. Unfortunately, this method was prone to bacterial contamination, villus atrophy, and epithelial hypertrophy, which limited their value [57]. Mutwiri et al. 1999, originally developed the gut-loop model in fetal lambs consisting of a single sterile intestinal 'loop' to assess functionality of the JPP and IPP to Ag [58]. Gerdts et al. 2001, using 4-6 month old sheep developed the model further by creating multiple intestinal 'loops' to create independent sites useful for the analysis of the induction of multiple mucosal immune responses in the small intestine [59].

1.11 Cytosine-Phosphate-Guanosine oligonucleotide (CpG ODN)

1.11.1 Introduction to CpG ODN

Our present day understanding about CpG ODN began to unfold over 100 years ago when scientists used extracts of bacteria to treat a variety of cancers, as reviewed in Wiemann and Starnes (2004) [60]. However, the elucidation of the immune stimulating nature of the CpG motif was not apparent until the era of rational drug design that involved the use of antisense ODNs to turn off target genes as reviewed in Krieg (2002) [61]. Eventually after numerous studies involving antisense ODNs it became clear through structure and function analysis that the stimulatory effect was not due to secondary structure or palindromes, but rather a consensus motif of XCGY, where X is any base but C, and Y is any base but G [61]. Interestingly the bases flanking the CpG dinucleotide

determine how immunostimulatory a CpG ODN will be in a given species [61]. For example, in mice the best immunostimulatory CpG motif is GACGTT, whereas in humans the optimal motif is GTCGTT, which is also immunostimulatory in a number of other species such as cows, sheep, chickens, cats, and dogs [62, 63]. Another important facet regarding the immune enhancing nature of CpG ODN is the composition of its backbone. For example, a phosphodiester backbone is susceptible to attack by nucleases reducing its half-life in the cell making it less immunostimulatory [64]. On the other hand CpG ODN composed of a phosphorothioate (sulfur replaces one of the non-bridging oxygen atoms) and or a mixture of phosphorothioate/phosphodiester backbone are much more resistant to nuclease attack thereby increasing the ODNs half life and hence its immunostimulatory capacity [64].

1.11.2 Cellular recognition of CpG ODN

The innate immune system with the help of pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), allow for the detection of various types of pathogens. Many microbes express markers called pathogen-associated molecular patterns (PAMPs) that are recognized by TLRs [65]. For example, TLR1 in conjunction with TLR6 as well as TLR2 recognize microbial lipopeptides [66-68]. TLR3 recognizes viral dsRNA, TLR4 is the receptor for LPS, TLR5 recognizes flagellin, TLR7 and TLR8 are implicated in viral-derived ssRNA, and TLR9 recognizes CpG DNA [69-74]. Thus, the TLR family members recognize specific patterns of microbial components. TLRs are type 1 transmembrane proteins conserved between insects and humans which contain an extracellular portion characterized by a variable number of leucine rich repeats [69]. The

cytoplasmic domains of TLRs are homologous to the mammalian intrerleukin-1 (IL-1) receptor and are known as the Toll-IL-1 R (TIR) domains [69]. Specifically, once CpG ODN is taken up into the endosome in a sequence non-specific process it signals via TLR9 after endosomal maturation [71].

1.11.3 Signaling through TLR9 pathway

Signaling by TLR9 is initiated through its TIR-domain in a MyD88 dependent pathway [72]. MyD88 binds to the cytoplasmic portion of TLR9 through its interaction with individual TIR domains, which in the case of CpG stimulation would be the TIR of TLR9 expressed in a late endosome [65, 73, 74]. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) of which 1 and 4 are critical in recruiting the next downstream molecule tumor necrosis factor receptor (TNFR)-associated factor 6, (TRAF6) [75]. TRAF6 activates downstream molecules that results in inhibitor of nuclear factor kappa B (IκB) degradation, allowing nuclear factor-kappa B (NF-κB) to move to the nucleus resulting in the activation of proinflammatory cytokines and several co stimulatory molecules [76].

1.11.4 Cells which respond to CpG ODN stimulation

CpG ODN stimulate a variety of murine and human leukocytes for example, B cells, bone marrow derived DC (BM-DC), monocytes/macrophages, and NK cells [60, 77-82]. T cells do not contain TLR9 and thus respond indirectly to CpG ODN if their T cell receptor (TCR) is cross linked first, i.e. as was demonstrated when CpG ODN was used in conjunction with anti-CD3 [83]. Unlike mice, humans have a small subset of cells that contain TLR9 and thus can respond directly to CpG ODN; these are plasmacytoid

dendritic cells and B cells [84, 85]. Two distinct classes of CpG ODN clusters have emerged as defined by their ability to preferentially stimulate either plasmacytoid dendritic cells (pDCs) or B cells in humans [86, 87]. Class A ODNs stimulate pDCs to secrete IFN-α and support NK cell production of IFN-γ [86]. Class B ODNs activate monocytes/dendritic cells to produce IL-6 and B cells to proliferate and secrete IgM [87]. A third class known as class C ODN has stimulatory properties of both classes and stimulates B cells to secrete IL-6 and pDCs to produce IFN-α [88]. Various farm animals also respond to CpG ODN stimulation [89, 90]. For example, PBMCs from pigs respond optimally to certain CpG motifs by increasing their expression of IL-6, IL-12, TNF-α, and IFN-γ [63, 91, 92]. Therefore, if an optimal porcine CpG ODN motif is used, pig leukocytes will respond, thus making pigs a good animal model to study the effects of CpG ODN.

1.11.5 Cytokines induced by CpG ODN

The type of cytokines induced by CpG stimulation varies depending upon the type of immune cell that is stimulated. In mice CpG ODN causes B cells to produce IL-6 and IL-10 [81, 93]. Mouse DCs and macrophages produce IL-12, IL-6, and TNF-α, with DCs primarily producing IL-12, and in macrophages IL-6, and TNF-α production predominates [71, 94]. Both T cells and NK cells in mice indirectly respond to CpG ODN by producing IFN-γ, which is also the only cytokine produced by human NK cells after CpG stimulation [80, 83, 95]. Human B cells secrete IL-6, whereas pDCs primarily produce IFN-α [62, 82] . In pigs the cytokines produced by CpG ODN stimulation of pDCs and B cells are not unlike those seen in their human analogs in that pDCs produce IFN-α, and B cells produce IL-6 [91, 96]. Besides the secretion of cytokines, CpG ODN also stimulated the

production of increased levels of MHC class II and the co stimulatory molecules B7-1 and B7-2 on both DCs and B cells [60, 97]. Thus by incorporating the adjuvant CpG ODN in our gut-loops in conjunction with the F4 Ag, the potential exists to enhance B cell proliferation and therefore the production of F4-specific IgA [98].

1.11.6 Adjuvanting effect of CpG ODN

It has been demonstrated that CpG ODN is immunostimulatory for B cells, pDCs, monocytes/macrophages, NK cells, and T cells in mice and humans [60, 62, 71, 80-83, 94, 95]. Numerous studies have also shown that farm animals or PBMCs from these animals respond to CpG ODN [89, 90, 99]. In pigs, CpG ODN has been shown to induce PBMCs to proliferate and secrete cytokines and interferons [100, 63, 91, 92]. Alcon *et al.* 2003, by co-delivering the outer membrane lipoprotein A of *Actinobacillus pleuropneumoniae* (*App*) with CpG ODN 2007 as an adjuvant in a novel lipid based formulation, elicited protection in pigs against infection with *App*. In other studies, the CpG motif ATGCAT has been shown to be mitogenic for pig B cells and effective as an adjuvant in a DNA-vaccine against pseudorabies virus (PrV) that provided protection against lethal PrV-infection [101].

1.12 Cationic antimicrobial peptides (AMPs)

1.12.1 Defensins and cathelicidens

AMPs are present in a variety of animal species. In mammals for example, two abundant classes are the defensins and cathelicidens [102]. Cathelicidens are a structurally diverse group of peptides with the exception of containing an N-terminal cathelin and C-

terminal microbicidal domain [64, 103]. Defensins on the other hand are all structurally similar in that they possess six invariant cysteine residues that form three intramolecular disulphide bonds [104]. Both cathelicidins and defensins are antimicrobial for a number of pathogens and expressed by a variety of tissues including neutrophils [105-107]. Moreover, they are chemotactic for a small subset of leukocytes, thus believed to link the innate with the adaptive immune response [108-110]. However, defensins unlike cathelicidins, do not bind endotoxin, are abundant in the small intestine, and are chemotactic for iDCs [106, 110].

1.12.2 Defensins: Classes, structures, and properties

Defensins can be subdivided into three classes α, β, and θ, based upon the position of their intramolecular disulfide bonds. For example α-defensins contain 3 intramolecular disulfide bonds between conserved cysteines in positions 1-6, 2-4, 3-5, and are *alpha* helical in structure [111]. The β-defensins on the other hand contain β sheet elements with cysteine bonds in positions 1-5, 2-4, and 3-6, whereas θ-defensins also contain three cysteine bonds but are cyclical in nature [112, 113]. Defensins range in size from 29-35 amino acids for α -defensins, up to 47 amino acids for β-defensins, and 18 amino acids for θ-defensins [113-115]. Defensins are amphipathic (hydrophilic and hydrophobic properties) molecules that contain several arginine and lysine residues that impart a net positive charge on the molecule [114, 116]. These properties make defensins microbicidal allowing them to bind and subsequently penetrate negatively charged bacterial membranes resulting in their lysis [117]. Defensins are active at concentrations ranging from 1-100 ug/ml, but all classes of defensins except θ-defensins have reduced antimicrobial activity at physiological concentrations of salt [117].

1.12.3 Recognition of β-defensins and consequences of their release

Defensins aside from their direct antimicrobial activity behave like chemokines. Specifically human β-defensins 1 and 2 are chemotactic for both human resting memory T cells and iDCs by interacting with chemokine receptor 6 (CCR6) [110]. Moreover they cause iDCs to mature thus promoting more effecient Ag presentation at inductive sites [118]. Therefore, β-defensins by facilitating the recruitment of a number of leukocytes notably iDCs, to the site of infection make them a natural adjuvant to be used in our gut-loops [118, 119].

1.12.4 β-Defensin as an adjuvant

The defensins innate property to act as an adjuvant has been used to enhance the immune response to a number of academic Ags, and human immunodeficiency virus-1 glycoprotein 120 (HIV gp120) [120-122]. Specifically, Biragyn et.al demonstrated that an HIV gp120 specific mucosal, systemic, and cytotoxic T lymphocytes (CTL) immune responses could be achieved against HIV gp120 by using a plasmid vaccine containing a β-defensin fused to HIV gp120 [120]. Thus, it has been shown that β-defensins act as adjuvants to enhance Ag specific immunity in the small intestine and are chemotactic for iDCs. Therefore, we used pBD-1 to adjuvant the F4-specific immune response in our gut-loops.

1.12.5 pBD-1 location and expression

The porcine β-defensin-1 is a small antimicrobial peptide of 42 amino acids in length that is produced as a 64 aa prepropeptide requiring cleavage in order for activation

to occur [123]. It is expressed in a variety of tissues such as the tongue (highest mRNA levels), respiratory and gastrointestinal tract [124]. Although developmentally regulated, pBD-1 was originally believed to be constitutively expressed however, it has recently been shown that its expression is inducible after bacterial infection [125]. It is microbicidal against a number of pathogens but high physiological salt concentrations like that seen in cystic fibrosis (120-170 mM) halt its activity [126]. Interestingly pBD-1 resembles human β-defensin-2 (HBD2) in its amino acid sequence and sites of expression [123]. The HBD2 is known to induce the migration of iDCs and memory T cells [110].

3. THE RESEARCH OBJECTIVES AND HYPOTHESIS

ETEC represent an important cause of neonatal and PWC, both of which are responsible for significant economic losses to the swine industry. ETEC strains causing PWC express a virulence factor known as the F4 fimbriae, which is used for adherence and subsequent colonization that leads to diarrhea. At weaning the piglet is susceptible to diarrhea because maternal antibodies are no longer available to the piglet; therefore active immunization is required to induce protective immunity. Moreover the induction of an immune response in the small intestine is required for protection against the disease and therefore oral vaccines that can induce mucosal immunity in the intestine are required. Several vaccination strategies have been tried in young piglets including parenteral immunization with live attenuated or inactivated ETEC F4, and edible vaccines such as alfalfa expressing the F4 Ag. With the exception of immunization with purified F4 fimbrial protein, most of the approaches have failed to provide protection against ETEC F4 challenge infection.

Interestingly, the induction of F4-specific mucosal immunity using the F4 appears to occur in $F4R^{pos}$ pigs only. Thus, a more detailed analysis of the induction of F4-specific local immunity in the small intestine is needed. Here we used an intestinal-loop model to investigate the induction of F4-specific immune responses in the intestine of post-weaned pigs. Surgically created jejunal loops were injected with crude F4

fimbrial protein and local immune responses were assessed three weeks later by quantifying the number of F4-specific ASCs isolated from PPs and gut-wall in the loops. By using the gut-loop model we were able to analyze multiple doses and vaccine formulations within the same animal reducing the variability associated with a large out-bred animals such as the pig.

The four objectives of this study including the hypothesis are listed below:

1) The first objective was to elicit an F4-specific immune response in surgically created gut-loops of three to four week old pigs. To this end loops were injected with purified F4 fimbriae and immune responses against the F4 fimbrial protein were analyzed by isolating the F4-specific ASCs from PPs and gut-wall in our loops. A modified ELISPOT assay was used to determine the number of F4-specific ASC that were present per 5 x 10^6 cells. Serum was collected on days 0, and 21, and F4-specific serum IgG was analyzed for using an ELISA.

2) The next objective was to investigate whether or not increasing the dose of the F4 protein would result in increased F4-specific immune responses in the gut-loops. Three doses of the F4 protein 500 μg, 250 μg, and 50 μ/loop were injected into separate loops to test this hypothesis. The ELISPOT was used to measure the F4-specific Ig ASC in the loops, and F4-specific responses in the serum were analyzed using ELISA.

3) Failing to see an increase in the F4-specific immune response due to changes in the dose we hypothesized that the local immune response in the small intestine could be enhanced by co-administration of the F4 with the adjuvant CpG ODN 2007 into the loops. This enhancement would be attributable to CpG ODN improving B-cell function

in the lamina propria and PPs of our loops as well as recruitment of more iDCs to the loops.

4) Lastly, we tested the adjuvant pBD-1 co-administered with the F4 protein into our gut- loops. We hypothesized that pBD-1 would enhance the F4-specific immune response in the loops by attracting iDCs and hastening their maturation.

3. THE IMPORTANCE OF THE F4 RECEPTOR IN RECEPTOR ELICITING MUCOSAL IMMUNE RESPONSES AGAINST *ESCHERICHIA COLI* IN PIGS

3.1 Abstract

ETEC F4 is an important pathogen in both humans and animals. In pigs, ETEC F4 causes post-weaning colibacillosis (PWC), a disease responsible for significant economic losses to global swine production. The development of clinical disease depends on the presence of the F4 fimbriae receptor (F4R) on epithelial cells in the intestine and only pigs that express the F4R display clinical disease. Effective prevention depends on the induction of local immunity in the small intestine. However, mucosal vaccines against PWC are currently not available and treatment is limited to the use of antibiotics. Here, we analyzed the role of the F4R for the induction of local immune responses in an *in vivo* gut-loop model in pigs. Various doses of purified F4 were administered into surgically created intestinal-loops and local immune responses were assessed by determining the number of F4-specific IgA- and IgG-ASC. In addition, the effect of the adjuvants CpG ODN and pBD-1 were assessed for their immunopotentiation of the crude F4 protein in our loops. The F4-specific immune responses were detected in immunized loops from both $F4R^{pos}$ and $F4R^{neg}$ animals. However, the magnitude of the immune response was dependent on the level of F4R-expression and was significantly stronger in $F4R^{mod\ to\ high}$ pigs compared to $F4R^{neg\ to\ low}$ pigs. Thus, our results demonstrate

that the expression level of the F4R is the most important factor for successful induction of mucosal immune responses against ETEC F4 infections in pigs.

3.2 Introduction

ETEC represent an important cause of neonatal and PWC, both of which are responsible for significant economic losses to the swine industry [1]. ETEC strains causing PWC express virulence factors known as fimbriae (F4, F18, 987P) and enterotoxins (LT, ST, EAST-1), which are responsible for bacterial adherence and diarrhea, respectively [1]. F4 fimbriae are proteinaceous bacterial appendages, which are composed of major (FaeG) and minor subunits (FaeC, FaeF, FaeH, and FaeI) [20]. FaeG mediates binding to the F4R, a group of glycoconjugates expressed on intestinal epithelial cells. Binding to the F4R is a requirement for bacterial adherence to the intestinal surfaces, and as a consequence, PWC occurs only in $F4R^{pos}$ pigs but not in $F4R^{neg}$ pigs [2-6]. Several strategies to prevent ETEC infections in pigs have been developed including maternal immunization, vaccination with live attenuated or inactivated bacteria, treatment with antibiotics, and selective breeding of $F4R^{neg}$ pigs [7, 8]. Although maternal immunization represents an effective means of controlling colibacillosis in neonatal pigs, sufficient levels of maternal antibodies are no longer available in post-weaned pigs. Thus, active immunization of the newborn piglet is required to induce protective immunity against PWC [9, 10]. However, effective vaccination must induce a mucosal immune response in the small intestine, as high levels of SIgA are required to protect against disease. For example, intramuscular immunization with purified F4 in combination with 1α, 25-dihydroxyvitamin D_3 induced strong systemic immune responses in $F4R^{pos}$ pigs, but the

induction of local immunity, and especially the induction of secretory IgA (SIgA) at the intestinal surfaces, was not achieved to any degree. Consequently, pigs were not protected against subsequent challenge infection [11]. In contrast, van den Broeck *et al*. (1999) showed that oral vaccination with purified F4 resulted in protection against challenge infection, demonstrating that the induction of mucosal immunity is critical for protection against the disease. However, the induction of protective mucosal immunity is complicated by a number of factors including the fact that only $F4R^+$ pigs responded to vaccination [12]. In addition, several factors such as genetics, nutritional status, and stress can play an important role in the disease outcome and further complicate the development and evaluation of vaccines [13]. Various strategies for mucosal vaccination against ETEC F4 have been tested including immunization with purified F4, recombinant FaeG monomers, edible F4 vaccines, incorporation of F4 into enteric-coated pellets, and immunization with attenuated live bacteria [3, 5, 12]. However, since most of these vaccination strategies induced only partial protective immunity, a more detailed analysis of the induction of F4-specific local immunity in the small intestine is needed. Here, we used an intestinal-loop model to investigate the induction of F4-specific immune responses in the intestine. Surgically created jejunal loops were injected with crude F4 fimbrial protein and local immune responses were assessed three weeks later by quantifying the number of F4-specific ASCs. Our gut-loop model facilitated the analysis of multiple immune responses within the same animal and therefore reduced the variability in results contributed by individual pigs [14]. Consequently, this model allowed testing of different doses and vaccine formulations within the same animal and same F4R background. Using this model, our objectives were to evaluate the role of the F4R during

the induction of F4-specific immunity in the small intestine, to identify a dose that elicits the strongest F4-specific immune response, and to analyze the effect of two potential mucosal adjuvants, namely CpG ODN and a chemically synthesized porcine beta-defensin 1 (pBD-1). Both adjuvants were chosen because of previous evidence for adjuvant activity in various animal models [15-19].

3.3 Materials & Methods

3.3.1 Isolation of crude F4 fimbriae

The *E. coli* isolate JG280, serotype 0149:H10:F4ac, $LT^{+}STaSTb$ (obtained from Dr. Carlton Gyles, U. of Guelph) was cultured with shaking (85 rpm) in tryptone soy broth (Difco Laboratories, Sparks MD USA) at 37^{0} C for 18 h. Bacteria were collected by centrifugation (4096 *g*, 15 min) followed by washing with phosphate-buffered saline (PBSA; 150 mM, pH 7.4), and further centrifugation at 4096 *g* for 15 min. As described by van den Broeck et al. (1999) the F4 fimbriae were isolated by homogenization of the resuspended bacteria using a Polytron® pt 3100 (Kinematica, AG) at 24000 rpm for 20 min at 4^{o}C. Centrifugation at 8074 *g* for 20 min and 16846 *g* for 40 min were carried out to remove large fragments. The solubilized fimbriae, present within the supernatant were precipitated with 40% ammonium sulfate overnight. The precipitate was centrifuged at 16846 g for 40 min, dissolved in Tris-HCl (100 mM, pH 7.4) plus 2M urea and subsequently dialyzed overnight against ultra-pure water to yield the crude F4 protein. The protein concentration of the isolated crude F4 was determined by the bicinchoninic acid reaction using bovine serum albumin as a standard (MJS Biolynx Inc., Brockville, Ontario). Purity of the crude fimbrial preparation was assessed by electrophoresis on 12%

SDS-PAGE gel and by western blot using a polyclonal rabbit anti-F4 serum (obtained from Dr. John Fairbrother, University of Montreal, Canada).

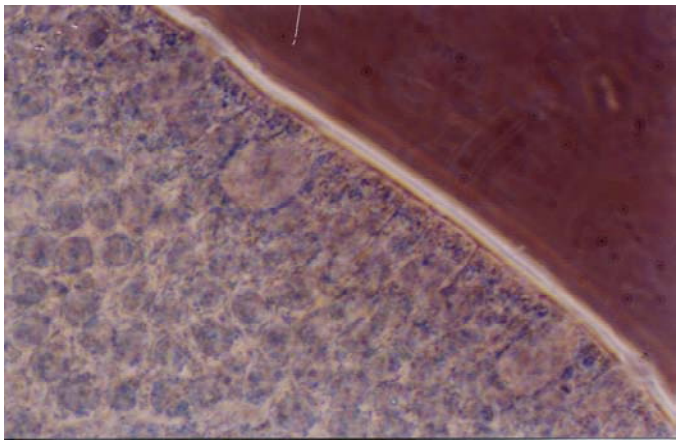
3.3.2 Production of pure F4 protein

Twenty-five ml of crude F4 fimbriae derived from the *E. coli* isolate JG280, serotype 0149:H10:F4ac, LT^{+}STaSTb using the mechanical shearing method, was subject to purification by SDS PAGE and electroelution. Two (16 x 20 cm) 12% acrylamide resolving gels were placed into a Large Format Vertical Electrophoresis chamber (Bio-Rad Laboratories, Mississiuaga, Ontario, Canada) and loaded with 12.5 ml of crude fimbrial protein. A voltage of 200 for 8 hours was required to separate the F4 protein into a discrete band. The gels were subsequently stained with Coomassie Brilliant Blue staining solution (EM SCIENCE, Gibbstown, New Jersey, USA) and then immediately destained. The gels were then placed onto 20 x 20 cm glass plates and the F4 protein bands approximately 2 x 20 cm in size, distinguishable from all other bands due to their width and deeper staining, were then cut away from each gel with a scalpel and subsequently cut into six 2 x 5cm strips and placed into the Elutrap device sample chamber with Tris-glycine (25mM Tris, PH 8.3; 192mM glycine; 0.1% Sodium Dodecyl Sulphate) buffer. A voltage of 200 was applied for 4 hours, after which the eluate was collected and the amount of the pure F4 protein determined by the bicinchoninic acid reaction using bovine serum albumin as a standard (MJS Biolynx Inc., Brockville, Ontario, Canada). SDS PAGE and WESTERN Blot analysis confirmed the purity. The eluted F4 protein was immediately stored at -80^{o}C.

3.3.3 *In vitro* villous adhesion assay

In order to determine the presence of the F4R on the mucosal epithelium, IVVAs were performed as described before [12]. Fifteen cm sections of jejunum were excised at the time of surgery. Collected segments were immediately processed to obtain the villi. The segments were opened along the mesentery and washed in PBSA. The villi were scraped off into Krebs–Henseleit buffer containing 1% formaldehyde at 4°C and used immediately or stored at 4°C overnight. Villi were washed in Krebs–Henseleit buffer without formaldehyde (at 4°C) before 4×10^8 ETEC F4 were added to an average of 50 villi in 0.5 ml PBS with 1% D-mannose and incubated at RT for 1 h while gently shaking at 80 rpm. Adherence of the bacteria to the villi was examined by phase contrast microscopy at a magnification of 40x and 100x and determined by counting the number of bacteria adhering along a 50 um length of villous brush border at 20 different places of at least five different villi, after which the mean bacterial adhesion per 250 um villous lengths was calculated. Adhesion of more than 40 bacteria per 250 um villous was designated as $F4R^{high\ (+++)}$, 25-39 bacteria as $F4R^{medium\ (++)}$, 6-24 bacteria as $F4R^{low\ (+)}$, and less than five bacteria per 250 um villous length was regarded as $F4R^{neg}$ (see Figure 3.2)

A



B

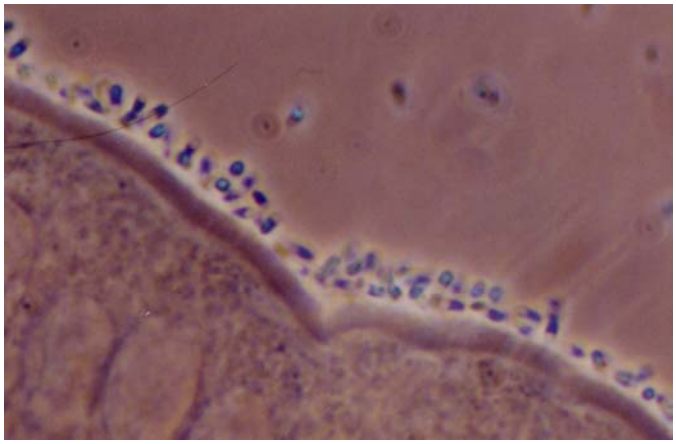


Fig 3.1. The *in vitro* villus assay (IVVA). Binding of ETEC F4 to a $F4R^{neg}$ pig (A) and a $F4R^{high}$ (B.) Area of approximately ~175 μm in length was assessed by phase contrast microscopy (1000x) for the presence of bacteria adhering to the brush border. Categorization: ($F4R^{high\ (+++)}$ > 40 adhering ETEC, $F4R^{med\ (++)}$ 25-39 adhering ETEC, $F4R^{low\ (+)}$ 5-24 adhering ETEC, $F4R^{neg}$ < 5 adhering ETEC.

3.3.4 The inhibition of ETEC F4 to F4-specific brush border receptors by F4ac fimbriae, F4ac-specific rabbit polyclonal, and mouse monoclonal antibodies

The IVVA used villi that had been scraped off from 15 cm jejunal sections of an $F4R^{pos}$ pig, and stored at $4^{o}C$ in Krebs–Henseleit buffer containing 1% formaldehyde. These villi were then washed in Krebs–Henseleit buffer without formaldehyde (at $4^{o}C$) of which aliquots of approximately 50 villi were added to separate reaction tubes containing 500 ul of PBS with 1% D-mannose plus one of the following amounts of F4ac fimbriae 0, 2, 10, 20, or 50 µl; and 2, 5, 10, or 20 µl, of F4 ac-specific polyclonal rabbit serum (rPAb) (obtained from F4 immunized rabbits at VIDO), and 2, 5, 10, or 20 µl, F4 ac-specific monoclonal mouse serum (mMAb) (Kindly provided by Frank Verdonk, Ghent University, Merelbeke, Belgium) [20]. An irrelevant mouse IgG antibody (Caltag Labs; Burlingame, California) and non-immunized rabbit serum were used as negative controls for the F4ac-specific mMAb and rPAb IVVA inhibition experiments respectively. These mixtures were shaken at 80 rpm at room temperature for 60 minutes, at which time 4×10^{8} ETEC F4ac bacteria were added and shaken at 80 rpm for one hour at room temperature. The adherence was determined by counting the number of bacteria adhering along a 50 um length of villous brush border at 20 different places from at least five different villi, after which the mean bacterial adhesion per 250 um villous lengths was calculated. The inhibition of ETEC F4 binding to villi by the various concentrations of F4ac protein was examined by phase contrast microscopy at a magnification of 1000x. The percentage adhesion was calculated by multiplying by 100 the ratio between

adhesion with and without F4ac pre-incubation. One hundred minus this adhesion percentage results in the inhibition percentage.

3.3.5 Animals, surgery, and post-surgical treatment

All animal experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Three to four week old pre-weaned piglets, obtained from the Prairie Swine Centre, University of Saskatchewan, were housed at the Vaccine & Infectious Disease Organization (VIDO) animal facility. The pigs were fasted for 12-18 h prior to surgery and pre-medicated by intramuscular injection of xylazine (2 mg/kg) and ketamine (20 mg/kg). Anesthesia was maintained using isoflurane. Intestinal segments were surgically created as described before [14]. Briefly, an intestinal segment containing six to eight Peyer's patches (PPs) was separated from the normal intestine, flushed with 100 ml of sterile phosphate-buffered saline without Ca^{++} and Mg^{++} (PBSA) to remove contents, and injected with 60 ml PBSA containing metronidazole (Abbot Labs Ltd, Saint-Laurent, Quebec, Canada) (250 mg) and enrofloxacine (Bayer Inc., Toronto, Ontario, Canada) (200 mg). In addition, a 15 cm long piece of jejunum was removed for the *in vitro* villous adhesion assay (IVVA). The patency of the non-segment intestine was re-established by an end-to-end anastomosis. The metronidazole and enrofloxacin solution was removed with a PBSA flush prior to closing both ends of the intestinal segment with an inversion type suture pattern. PP were macroscopically identified and silk ligatures were tied proximal and distal to each PP to create ~20 cm isolated segments containing a PP loop separated by interspaces of various lengths that lacked PP. All loops and interspaces were

injected with PBSA containing 1 mg of ampicillin (Sigma Aldrich Co). The F4 or the combination of F4 with either CpG ODN 2007 (obtained from Merial Limited, Duluth, Georgia) or the pBD-1 was injected in a volume of 1 ml PBSA into each loop. Control loops received 1 ml PBSA instead. The proximal loop was marked by an additional suture and a map was drawn for the identification of immunized and non-immunized control loops. The intestine was replaced into the abdominal cavity and the surgical incision in the abdominal wall was closed in three layers. Post-operatively, flunixin meglumine and enrofloxacin were administered for three and five days, respectively (see Figure 3.1).

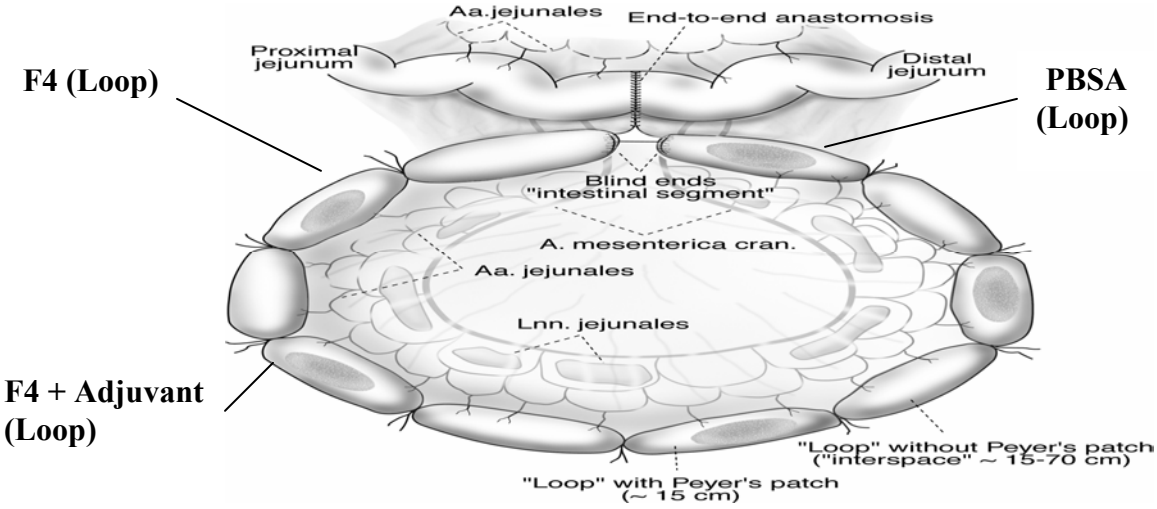


Figure 3.2. The Gut-loop model was adapted for use in 3-4 week old pigs (Gerdts et al, 2001), which allowed us to analyze the induction of the local immune responses against the F4 Ag alone or co injected with the adjuvants CpG and porcine β-defensin-1 (pBD-1) into loops with Peyer's patches. After 3.5 weeks, F4- specific ASC were isolated from the Peyer's patches or gut wall and analyzed using a modified ELISPOT assay.

3.3.6 Samples and tissue collection

To determine serum antibody titers against F4, serum was collected from each pig the day before surgery and at weekly intervals following surgery. The pigs were euthanized three weeks after surgery by lethal injection (Euthanyl, MTC Pharmaceuticals Ltd, Cambridge, Ontario) and intestinal loops and mesenteric lymph nodes were immediately placed into cold PBSA. Each loop was subsequently injected with 10ml PBSA + 250 µl of 100 mM phenylmethylsulphonylfluoride (PMSF). Loops were cut open along the mesenteric border and contents were collected into 50 ml centrifuge tubes (Corning, Corning, NY, USA). PPs were dissected from each loop and placed separately in 10 ml ice-cold PBSA containing 1% antibiotic/antimycotic (penicillin G sodium 10,000 units/ml, streptomycin sulfate 10,000 ug/ml, amphotericin B 25 ug/ml) (Gibco BRL, Burlington, ON, Canada). Contents were thoroughly mixed by vortexing with 10 ml PBSA and 200 ul 100 mM PMSF (Sigma-Aldrich, Oakville, ON, Canada) and particulate material was removed by centrifugation at 600 *g* for 15 min. Supernatants were stored at −70 °C.

3.3.7 Isolation of PP cells

Follicles and cells from the interfollicular region of the PP were obtained by scraping the mucosal surface from the underlying muscularis externa into PBSA + 0.1% EDTA (BDH, Toronto, Ontario). Large fragments of the mucosa were removed before subjecting the remaining follicles and cells to vigorous pipetting to create a single cell suspension [14]. This suspension was filtered through a 40 micron nylon mesh cell strainer

(Becton Dickinson Labware, NJ, USA), then subsequently washed in PBSA and resuspended in RPMI medium (Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 50 uM 2-mercaptoethanol (Sigma-Aldrich). All subsequent assays employed this medium. Using the trypan blue exclusion method, viable cells were counted using a Neubauer haemocytometer. Cells were resuspended in medium at a concentration of 5×10^6 cells/ml, which were subsequently used in the ASC assay.

3.3.8 Isolation of intraepithelial and lamina propria lymphocytes

Pigs were euthanized with an i.v. injection of pentobarbital sodium (Euthanyl, Bimeda, MTC, Cambridge, ON, Canada) and the gut-loops and interspaces were collected and placed immediately into cold PBSA containing antibiotic-antimycotic solution (Gibco, Invitrogen, Inc., Grand Island, NY, USA). Another intestinal portion of 15-20 cm was cut along the mesenteric border, blotted on paper towel and washed in PBSA. Subsequently, this intestinal segment was cut into small (0.5 cm^2) pieces and placed into a 50 ml Teflon-Beakers containing 25 ml of a solution containing 5% fetal bovine serum (FBS, SeraCare Life Sciences, Inc., Oceanside, CA, USA), 0.01M Hepes Buffer (Sigma, St Louis, MO, USA), 50 μg of Enrofloxacin, and 2 mM EDTA (EMD Chemicals, Gibbstown, NJ, USA). This solution was denominated PBSA-buffer-EDTA. Approximately 40 ml of non-segment small intestinal contents were collected in 50 ml tube. All these samples were kept in ice until processed.

The Teflon-beakers containing the intestinal pieces were incubated for 90 minutes on a magnetic multi-stirrer, set on moderate agitation, at 37˚C. The supernatant, which

contained intraepithelial lymphocytes and epithelial cells, was collected by filtering the tissues fragments and mucous through a plastic strainer. This filtered supernatant was centrifuged at 1200 rpm for 15 minutes at 4˚C. The pellets were re-suspended in PBSA-buffer without EDTA and centrifuged as above. This washing step was repeated twice. The remaining tissue fragments were incubated for 90 minutes in PBSA-buffer containing 250U/ml of collagenase type 1 (Worthington Biochemical, Lakewood, NJ, USA) at 37˚C and moderate agitation. After this incubation the supernatant containing lamina propria cells was filtered and cells were washed in the same way as the intra-epithelial cells. The intraepithelial and lamina propria population of cells were then pooled, centrifuged at 1200 rpm for 15 minutes, re-suspended in 30% Percoll (Amersham Biosciences, Uppsala, Sweden), and centrifuged for 20 minutes 1650 rpm at room temperature. The top layer of cells, which contained epithelial cells, was discarded and the pellet was re-suspended in PBSA-buffer and centrifuged for 10 minutes, 800 rpm, at 4˚C. The pellet was re-suspended in RPMI-supplemented, the cells were counted and plated in ELISPOT plates.

3.3.9 Antigen-specific ASC assay

Ag-specific antibody-secreting cells (ASC) were detected using a modified ELISPOT assay. 96 well nitrocellulose filtration plates (Whatman Inc, Clifton, NJ, USA) were coated overnight with either 50 μg/ ml of pure F4 fimbrial protein at 100 μl/well or 500 μg/ ml of chicken egg Ovalbumin (OVA) (SIGMA, St. Louis, MO, USA) at 100 μl/well . Wells were washed to remove free protein before adding 5×10^5 cells/well to triplicate wells, in a final volume of 200 ul culture medium. Cultures were incubated for

18 h at 37 °C in a humidified atmosphere with 5% CO_2 before removing the cells. ASC were detected by first adding mouse anti-porcine IgA (1:300 dilution; Cedarlane Laboratories, Hamilton ON, Canada) followed by phosphatase labeled goat anti-mouse IgG (H+L chain specific; 1:5000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) or phosphatase labeled goat anti-swine IgG (H+L chain specific; 1:5000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). ASC were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) insoluble alkaline phosphatase substrate (Sigma-Aldrich). The frequency of F4-specific ASC per 5×10^6 cells was calculated by subtracting the number of ASC detected in wells not coated with Ag from the number of ASC detected in Ag-coated wells. An inverted light microscope was used to count three replicate wells for each cell population isolated from a given loop, and data presented are mean values of replicate samples.

3.3.10 ELISA

Polystyrene microtiter plates (Immunolon® II; Dynatech Laboratories, Gaithersburg, Maryland, USA) were coated with 50 μg/ml of pure F4 fimbrial protein and incubated with serum (1 in 40) dilution 4 fold diluted). Phosphatase labeled goat anti-swine IgG (H+L chain specific; 1:5000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used to detect serum IgG, whereas mouse anti-porcine IgA (1:300 dilution; Cedarlane Laboratories, Hamilton ON, Canada) plus phosphatase labeled goat anti-mouse Ig (H+L chain specific; 1:5000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were used to detect F4-specific IgA secreted into

the loops. The reaction was visualized with *p*-nitrophenyl phosphate (PNPP; Sigma-Aldrich). The titer equals the reciprocal of the highest dilution that gave a positive OD reading. A positive reading was defined as an OD that was at least two times greater than the values for a negative sample.

3.3.11 Statistical analysis

Individual loops were treated as a unit of analysis (n = 113). A regression analysis was performed to determine the relationship between F4R-expression and the number of either IgA-ASCs or IgG-ASCs. In order to increase statistical power, all F4-immunized loops were included into the analysis (total of F4-immunized loops: n = 77; total of control loops: n = 36). The effect of F4R-expression, F4 dose and the interaction of these two variables upon the number of ASCs were examined using ordinary least squares regression. The IgA-ASC and IgG-ASC data were not normally distributed and therefore the logarithm of each variable was used in the regression analysis. *P* values less than 0.05 were considered significant.

3.3.12 Experimental outline

Five experiments were performed (see Table 3.1) to address our objectives and hypothesis.

Experiment 1, was performed to test the immunogenicity of our electroeluted purified F4 fimbrial protein in surgically created pig gut-loops in 3-week old pigs. OVA was injected into loops as a positive control, PBSA loops were our negative control loops

Experiment 2, was used to test the immunogenicity of the crude form of the F4 protein in surgically created pig gut-loops in 3-week old pigs. OVA was injected into loops as a positive control, PBSA loops were our negative control loops

Experiment 3 was a dose titration trial to determine the effect of dose on the F4-specific immune response in pig gut-loops in 3-week old pigs. PBSA loops were used as a negative control.

Experiments 4 and 5 tested our hypothesis that an adjuvant effect could be obtained by co injecting either CpG ODN 2007 or pBD-1 with crude F4 fimbrial protein into the gut-loops of 3-week-old pigs. The surgical maps of all gut-loop experiments are shown in (Figure 3.3)

Table 3.1. The experimental design for all five gut loop experiments that lists the number of pigs/trial, the concentration of F4 Ag, OVA, and adjuvant. The number of immunized and non-immunized loops are also shown.

Exp	# of Pigs	Dose of F4 (µg/loop)	Dose of OVA(µg/loop)	# of loops immunized	# of control loops immunized
1	4	500	500	4	2
2	4	500	500	4	2
3	5	50, 250, 500		6	2
Exp	**# of Pigs**	**Dose of F4 (µg/loop)**	**Dose of CpG (µg/loop)**	**# of loops immunized**	**# of Control loops immunized**
4	6	125	500	6	2
Exp	**# of Pigs**	**Dose of F4 (µg/loop)**	**Dose of pBD-1 (µg/loop)**	**# of loops immunized**	**# of Control loops immunized**
5	7	125	500	6	2

ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP
-	500 µg F4	-	500 µg F4	-	500 µg OVA	-	500 µg OVA	-	PBSA	-	PBSA

A. Experiments 1 & 2.

ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	Loop with PP	ISP	Loop with PP
-	50 µg F4	-	50 µg F4	-	250 µg F4	-	250 µg F4	-	500 µg F4	-	500 µg F4	PBSA	-	PBSA

B. Experiment 3.

ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	Loop with PP	ISP	Loop with PP
-	125 µg F4	-	125 µg F4	-	125 µg F4	-	125 µg F4 + 500 µg CpG	-	125 µg F4 + 500 µg CpG	-	125 µg F4 + 500 µg CpG	PBSA	-	PBSA

C. Experiment 4.

ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	ISP	Loop with PP	Loop with PP	ISP	Loop with PP
-	125 µg F4	-	125 µg F4	-	125 µg F4	-	125 µg C F4+ 250 µg pBD1	-	125 µg F4+ 250 µg pBD1	-	125 µg F4+ 250 µg pBD1	PBSA	-	PBSA

D. Experiment 5

Figure 3.3. Surgical maps: (A) and (B) tested the immunogenicity of the F4; (C) and (D) tested adjuvants CpG ODN and pBD-1.

3.4 Results

3.4.1 Purification of F4ac fimbriae using electro-elution

To obtain pure F4 protein, SDS-PAGE gels were loaded with crude F4 protein and afterwards a single band of 26kDa in size was electroeluted and named pure F4 protein. Purity was confirmed by SDS PAGE gel (Figure. 3.4 (A)) and was recognized by an F4-specific rabbit polyclonal antibody in our Western blot (Figure. 3.4 (B)). The electroelution process yielded approximately 2.5 mg of pure F4 fimbrial protein from 4 liters of ETEC F4 liquid culture. In contrast the mechanical shearing method used to obtain crude F4 protein yielded approximately 8-10 mg of F4 protein from 1 liter of ETEC F4 liquid culture.

3.4.2 Inhibition of ETEC F4ac to F4-specific brush border receptors by F4ac fimbrial protein

The use of mechanically sheared F4ac fimbriae to block the adhesion of ETEC F4ac to F4-specific brush border receptors of an $F4R^{pos}$ pig was tested against four different concentrations of F4ac protein at 2, 10, 20 and 50 μg (Figure 3.5). As the amount of F4 protein was increased from 2 to 50 μg the number of ETEC F4 adhering to villous brush border receptors decreased. The IVVA was used to measure the inhibition effect of the F4 protein on ETEC F4 binding to $F4R^{pos}$ villi.

No inhibition was seen when 0, 2, and 10 μgs of F4 protein were used as their mean adherence values were 32 ± 12, 31 ± 7, and 40 ± 11, coliforms binding / 250 μm of villous brush border respectively. Conversely at 20 and 50 μgs of F4 a marked decline in adherence was seen as evidenced by mean adherence values of 6 ± 2, and 0 ± 0, ETEC

F4 binding / 250 μm of villous brush border respectively. Thus a statistically significant effect was seen for F4 fimbriae to inhibit the adherence of ETEC F4 to F4R^{pos} villi ($P<0.0001$; $r^2= 57\%$) as the inhibition rose from 0% at 10 μg of F4ac protein, to 100% for 50 μg of F4ac fimbriae used.

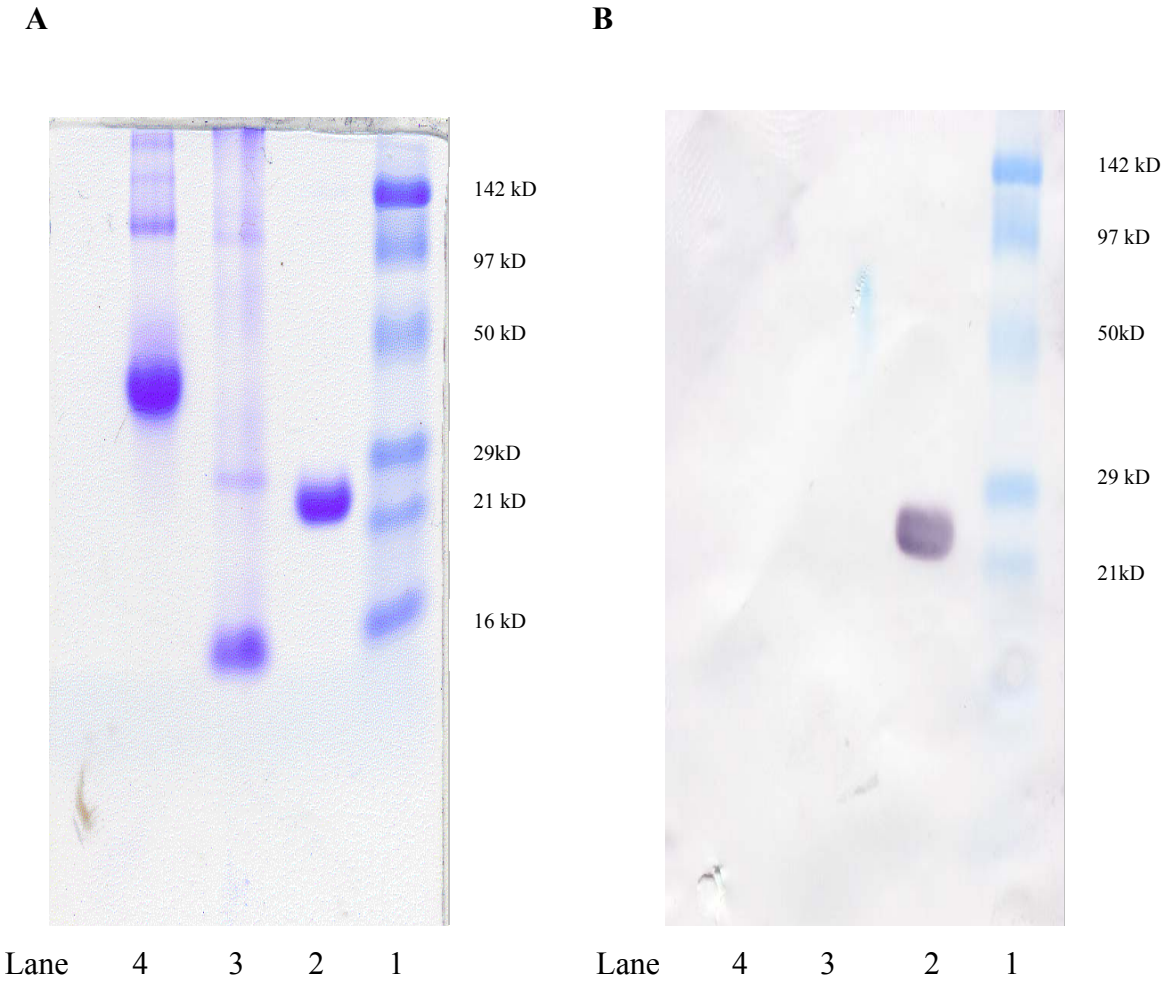


Figure 3.4 SDS PAGE and Western blot were used to demonstrate the purity of the F4 protein (26kD). The F4 fimbrial protein which was purified by SDS PAGE and electroelution is shown on an SDS PAGE gel (A); along with a non-fimbriated *E.coli* strain (EL14) that was processed by centrifugation, shearing, ammonium sulphate precipitation, and overnight dialysis in ultra-pure H_20. Five micrograms of protein was loaded per well; lane 1 marker; lane 2 pure F4; lane 3 EL14; lane 4 ovalbumin (OVA). The identification of F4ac fimbriae in a Western Blot (B); lane 1 marker; lane 2 pure F4; lane 3 EL14; lane 4 ovalbumin (OVA), a polyclonal serum derived from F4 crude-immunized rabbits was used for detecting the purified F4 fimbriae.

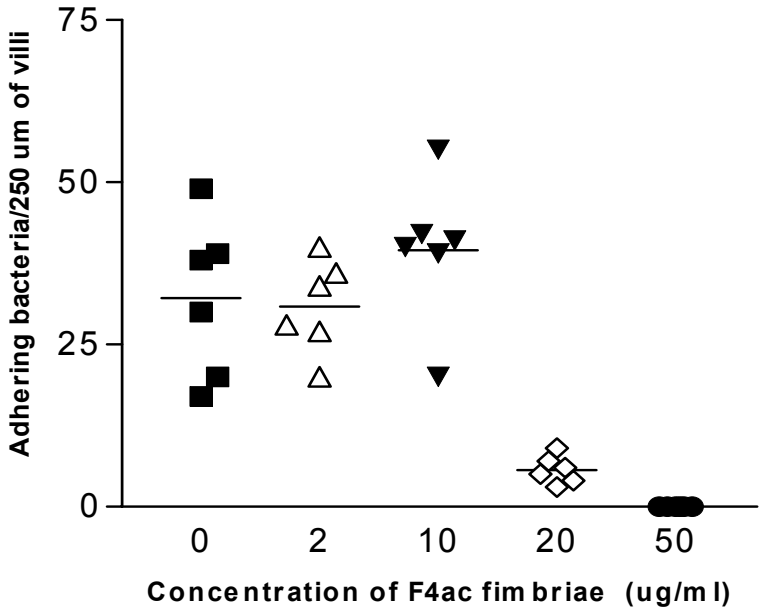


Figure 3.5 The inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig by pre-incubating the villi with various concentrations of F4ac crude fimbrial protein (2, 10, and 20, 50 µgs/ml) obtained by the shearing method. No inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig was seen when F4ac crude fimbrial protein was omitted in the pre-incubation step.

3.4.3 Use of rPAb to inhibit binding of ETEC F4ac to F4-specific brush border receptors

The use of rPAb to block the adhesion of ETEC F4 to F4-specific brush border receptors of an F4R^{pos} pig was tested against the following volumes of F4-specific rPAb 2, 5, 10, and 20 µls (Figure 3.6). As the amount of rPAb increased from 2 to 20 µl the number of ETEC F4 adhering to villous brush border receptors decreased. The IVVA was used to measure the inhibition effect of rPAb on ETEC F4 binding to F4R^{pos} villi.

There was a statistically significant effect of the rPAb to inhibit the binding of ETEC F4 to F4R^{pos} villi ($P<0.0001$; $r^2= 83\%$). Villi that were pre-incubated with 2 and 20 µl of

F4-specific rPAb had a mean adherence of 44 ± 9.3 (0% inhibition) and 3.0 ± 2.9 (93 % inhibition) coliforms binding / 250 μm of villous brush border respectively compared to a mean of 42 ± 4.4 adherent ETEC F4 / 250 um of villi when no F4-specific rPAb was used. Conversely there was no inhibition effect upon the adherence when preimmune rPAb was used (P=0.76; r^2= 0.05%). Villi that were pre-incubated with 2 and 20 μl rPAb had a mean adherence of 39.3 ± 11.7 and 44.5 ± 9.9 respectively.

3.4.4 Use of mMAb to inhibit binding of ETEC F4ac to F4-specific brush border receptors

The use of mMAb to block the adhesion of ETEC F4 to F4-specific brush border receptors of an $F4R^{pos}$ pig was tested against four different volumes of F4-specific mMAb 2, 5, 10, and 20 μls (Figure 3.7). As the amount of mMAb increased from 2 to 20 μl the number of ETEC F4 adhering to villous brush border receptors decreased. The IVVA was used to measure the inhibition effect of mMAb on ETEC F4 binding to $F4R^{pos}$ villi.

There was a statistically significant effect of the mMAb to inhibit the binding of ETEC F4 to $F4R^{pos}$ villi (P<0.0007; r^2= 46%). Villi that were pre-incubated with 2 and 20 μl of F4-specific mMAb had a mean adherence of 44.5 ± 4.9 (0% inhibition) and 5.0 ± 7.6 (87.5 % inhibition) coliforms binding / 250 μm of villous brush border respectively compared to a mean of 40.25 ± 20.7 adherent ETEC F4 / 250 um of villi when no F4-specific mMAb was used. Conversely there was no inhibition effect upon the adherence when mIAb serum was used (P=0.82; r^2= 0.05%). Villi that were pre-incubated with 2 and 20 μl mIAb had mean adherence values of 42.5 ± 8.7 and 39.0 ± 10.7 respectively.

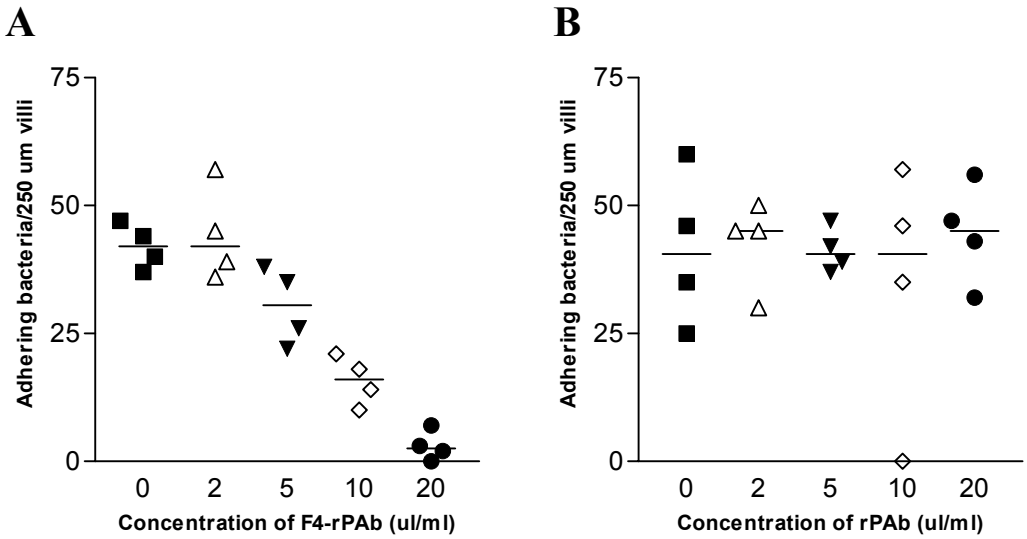


Figure 3.6 A: The inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig by pre-incubating the villi with various concentrations of F4ac-specific rabbit polyclonal antibody (rPAb) B: No inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig by pre-incubating the villi with various concentrations of an rabbit polyclonal antibody (rPAb) (used as a control).

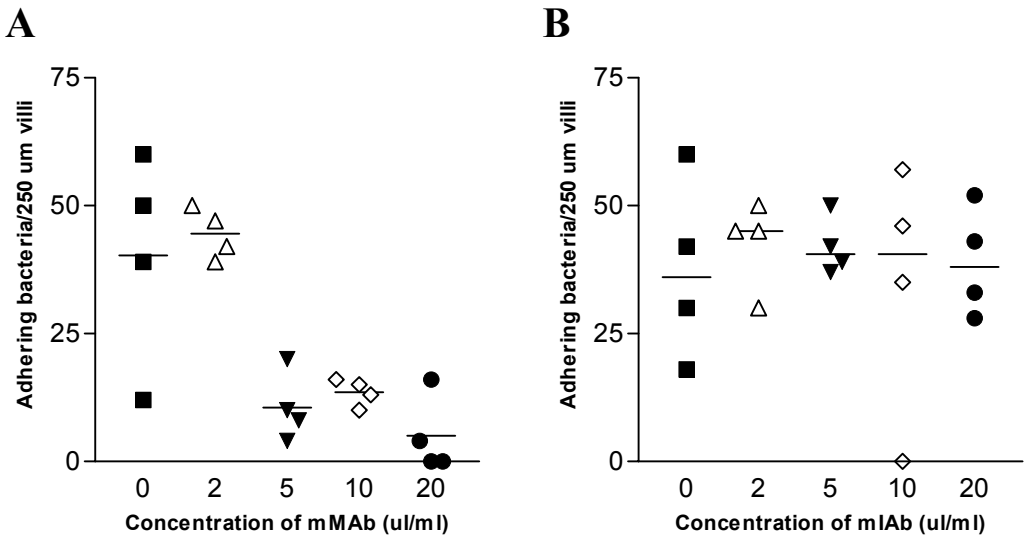


Figure 3.7 A: The inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig by pre-incubating the villi with various concentrations of F4ac-specific mouse monoclonal antibody (mMAb) B: No inhibition of adherence of ETEC F4 binding to villi of an F4R^{pos} pig by pre-incubating the villi with various concentrations of an irrelevant mouse polyclonal antibody (mIAb) (Mouse IgG1-specific) used as a control.

3.4.5 Induction of local intestinal immune responses against pure F4 fimbriae

Local immune responses against the electroeluted F4 fimbriae were assessed after 24 days by quantifying the number of IgA- and IgG-ASCs in PPs for each of the loops containing 250 and 500 ug of pure F4 protein. A detailed outline of the experimental design is given in table 3.2. No F4-specific ASCs were found in loops immunized with the pure F4 protein. Further, no F4-specific immune responses were found in PPs from PBSA-treated and OVA-immunized loops. OVA specific IgA- and IgG-ASCs were not elicited in OVA immunized loops neither.

3.4.6 Induction of local intestinal immune responses against crude F4 fimbrial protein.

Local immune responses against crude F4 fimbrial protein were assessed after 24 days by quantifying the number of IgA- and IgG-ASCs in PPs for each of the loops containing 500 ug of crude F4 protein. A detailed outline of the experimental design is given in table 3.2. Median values of 92 IgA-ASCs/ 5 x 10^6 cells (min. 6 – max. 274) and 55 IgG-ASC/ 5 x 10^6 cells (min. 3 – max. 216) isolated from PP were found (Figure 3.8). In contrast, no F4-specific ASCs were found in PPs from PBSA-treated and OVA-immunized loops. However, OVA specific IgA- and IgG-ASCs were found in loops immunized with 500 μg of OVA. Median values of 122 IgA-ASCs/ 5 x 10^6 cells (min. 10 – max. 308) and 58 IgG-ASCs/ 5 x 10^6 (min. 0 – max. 169) cells isolated from PP were found (Table 3.2).

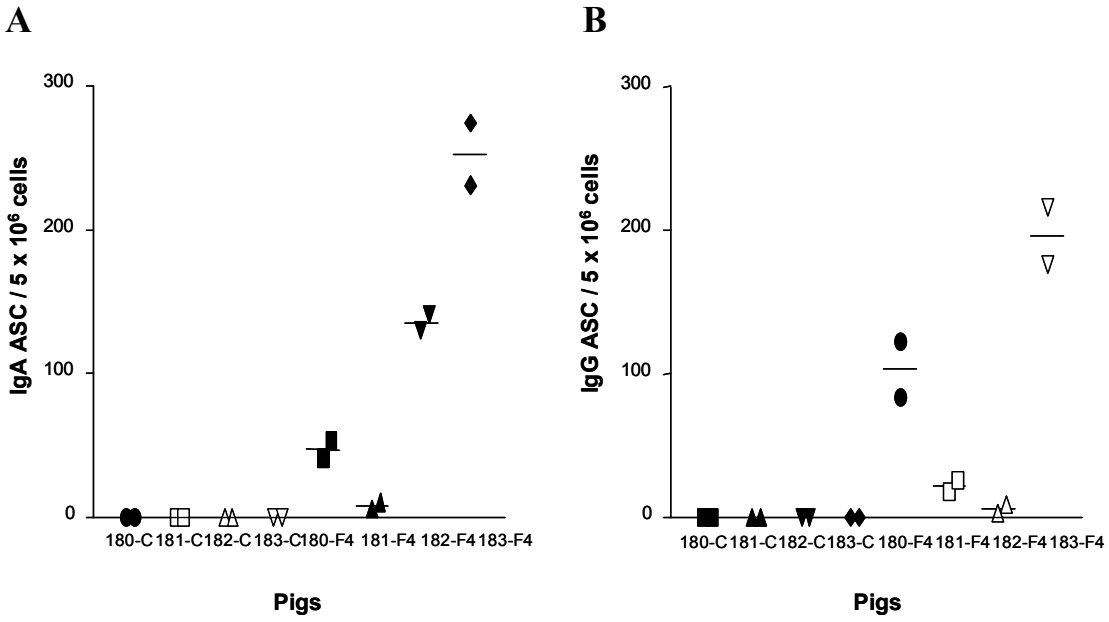


Figure 3.8 Number of F4-specific IgA-ASCs (A) and IgG-ASCs (B) in PPs from loops immunized with crude F4 protein. The number of F4-specific IgA- and IgG-ASC is shown. Each IgA- and IgG-ASC value is the mean ± the standard deviation (SD) of a triplicate of wells each receiving 100 µl of 5 x 10^6 isolated follicular PP cells/ml. The F4R statuses of the pigs were 180^{+++}, 181^{++}, 182^{+}, and 183^{+++}. Pig #-c represents the number of F4-specific Ig-ASCs in control loops; Pig #-F4 represents the number of F4-specific Ig-ASCs in F4-immunized loops.

Table 3.2 Number of F4- a5nd OVA-specific ASC in F4- and OVA-immunized loops. Non-immunized loops (not shown) had no F4-specific ASCs. $F4R^{high\ (+++)}$, $F4R^{medium\ (++)}$, $F4R^{low\ (+)}$, $F4R^{-\ neg}$.

Exp	Pig	F4 µg/loop	F4-ASC in immunized loops		OVA µg/loop	F4-ASC in OVA- immunized loops	
			IgA	IgG		IgA	IgG
1	74^{++}	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0
	75^{+++}	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0
	76^{-}	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0
	77^{++}	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0	500	0 ± 0; 0 ± 0	0 ± 0; 0 ± 0
2	180^{+++}	500	53 ± 16; 41 ± 8	84 ± 16; 123 ± 18	500	62 ± 1; 99±28	101 ± 26; 75 ± 5
	181^{++}	500	10 ± 3; 6 ± 2	18 ± 6; 26 ± 1	500	10± 2; 12 ±1	0 ± 0; 0 ± 0
	182^{+}	500	141 ±11; 130 ± 21	9 ± 7; 3 ± 3	500	307±39; 308 ± 57	17± 6; 22 ± 0
	183^{+++}	500	274 ± 15; 231 ± 33	216 ± 21; 176 ± 25	500	83 ± 4; 110 ± 12	169 ± 24; 82 ± 19

3.4.7 Immunization with varying doses of crude F4 protein

Five pigs were immunized with 50, 250 or 500 μg crude F4 protein in gut-loops and the number of F4-specific ASCs from the PPs were enumerated. A detailed outline of the experimental design is given in table 3.3. No F4-specific immune response was seen in the $F4R^{neg}$ pig at any dose. In contrast, the $F4R^{low}$ pig displayed between 1- 32 F4-specific IgA ASC/ 5 x 10^6 cells and 5 - 43 F4-specific IgG-ASC/ 5 x 10^6 PP cells (Figure 3.9 (B) and (D)). Between 6 - 146 F4-specific IgA-ASC/ 5 x 10^6 cells and 5 - 236 F4-specific IgG-ASC/ 5 x 10^6 cells were found in $F4R^{high}$ pigs depending on the dose used for immunization (Figure 3.9 (A-D)). With the exception of two loops in one animal, no F4-specific ASCs were found in PBSA-treated control loops (IgA-ASC: min. 0 – max. 4; IgG-ASC: min. 0 – max. 42). Due to the low number of pigs used in this dose experiment we felt that an analysis of all the loops from the four successful gut-loop experiments in which doses of 50, 125, 250 and 500 μgs of F4/loop were used would be more powerful in terms of statistical relevance regarding the dose effect. Therefore, the data obtained from all these experiments was subject to a least squares linear regression analysis to study the effect of dose, the expression level of the F4R, and level of maternal antibodies present in the serum, on the magnitude of the F4-specific log-IgA and IgG-ASC immune response (see appendix).

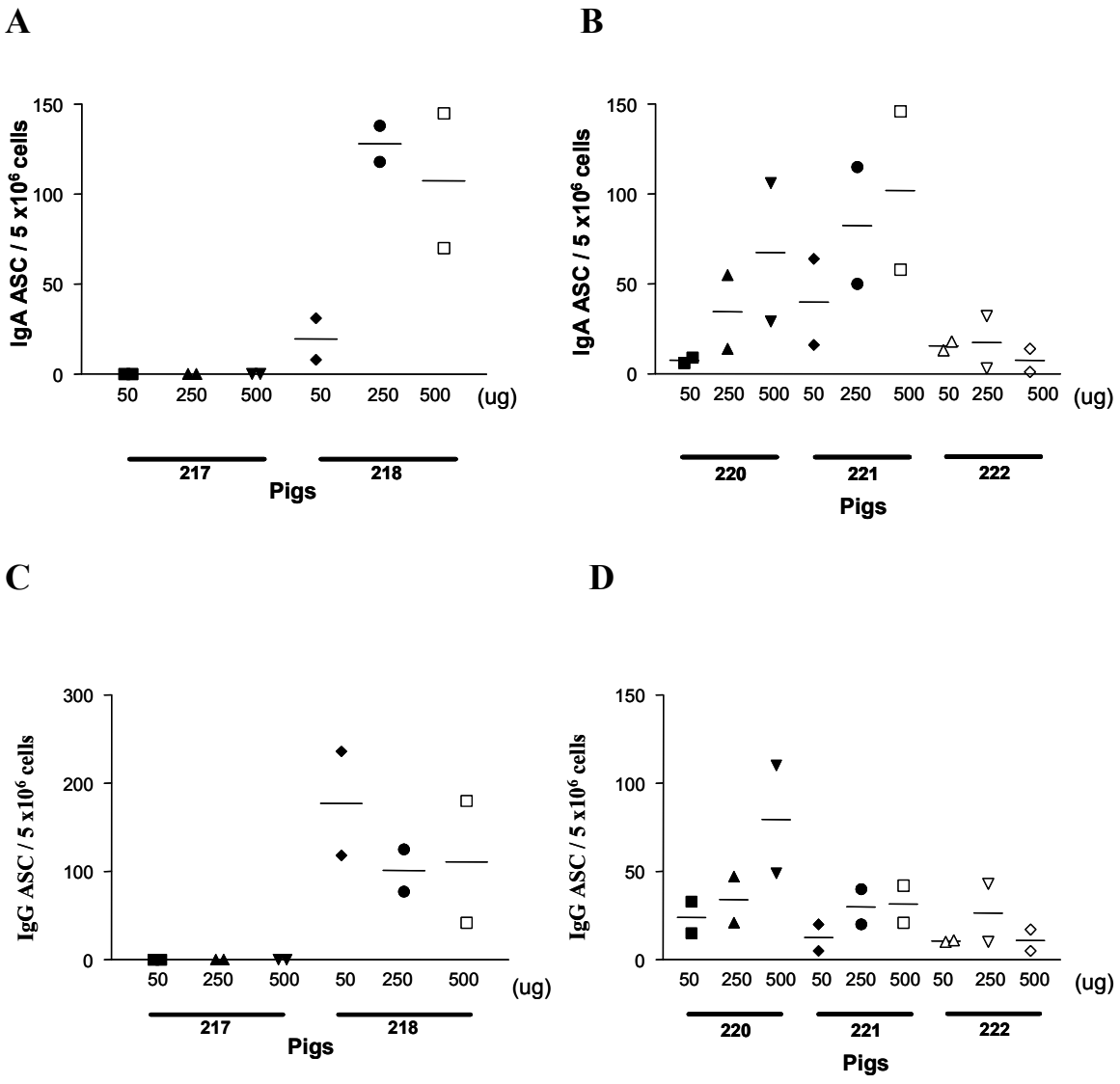


Figure 3.9 The effect of dose on the local F4-specific immune response against crude F4 fimbrial protein in pig gut-loops. Control loops were administered 1 ml of PBSA (not shown), and the immunized loops contained 500, 250 and 50 ug/ml of crude F4 fimbrial protein. (A) and (B) show the F4-specific IgA-ASC immune response, (C) and (D) the F4-specific IgG-ASC immune response in the loops from pigs 217, 218, 220, 221, and 222; Each IgA-ASC value is the mean ± the standard deviation (SD) of a triplicate of wells each receiving 100 µl of 5 x 10^6 isolated follicular PP cells/ml. The F4R statuses of the pigs were 217^{neg}, 218^{+++}, 220^{+++}, 221^{+++}, 222^{+}.

Table 3.3. Number of F4-specific ASC in F4-immunized and non-immunized control loops. $F4R^{high\ (+++)}$, $F4R^{medium\ (++)}$, $F4R^{low\ (+)}$, $F4R^{-\ neg}$.

Exp	Pig	F4 µg/loop	F4-ASC in immunized loops		F4-ASC in non-immunized loops (PBSA)	
3			IgA	IgG	IgA	IgG
	217-	50	0 ± 0 0 ± 0	0 ± 0 0 ± 0	0 ± 0	0 ± 0
		250	0 ± 0 0 ± 0	0 ± 0 0 ± 0	0 ± 0	0 ± 0
		500	0 ± 0 0 ± 0	0 ± 0 0 ± 0	0 ± 0	0 ± 0
	218^{+++}	50	31 ± 7 8 ± 5	236 ± 9 118 ± 54	0 ± 0 0 ± 0	42± 6 11 ± 3
		250	138 ± 13 118 ± 28	125 ± 17 77 ± 19		
		500	145 ± 11 70 ± 13	180 ± 19 42 ± 7		
	220^{+++}	50	6 ± 1 9 ± 1	15 ± 6 33 ± 1	0 ± 0 0 ± 0	0 ± 0 0 ± 0
		250	55 ± 14 14 ± 3	47 ± 10 21 ± 8		
		500	106 ± 20 29 ± 4	110 ± 19 49 ± 2		
	221^{+++}	50	64 ± 4 16 ± 2	20 ± 2 5 ± 2	0 ± 0 0 ± 0	0 ± 0 0 ± 0
		250	50 ± 4 115 ± 14	20 ± 7 40 ± 7		
		500	58 ± 8 146 ± 7	21 ± 1 42 ± 8		
	222^{+}	50	13 ± 6 18 ± 7	10 ± 3 11 ± 2	0 ± 0 0 ± 0	0 ± 0 0 ± 0
		250	32 ± 8 3 ± 1	43 ± 11 10±2		
		500	1 ±1 14 ± 1	5 ± 3 17 ± 2		

3.4.8 The adjuvant CpG ODN did not enhance the local F4-specific immune response in loops when coadminstered with crude F4 protein

From our dose experiment, we determined that immunizing with either 250 μg or 500 μg crude F4 protein on average induced higher F4-specific ASC-immune responses in the loops of $F4R^{pos}$ pigs than immunization with the dose of 125 μg F4/loop. Therefore 125 μg F4/loop was chosen to be co administered with CpG ODN so that the effect of adjuvant could be detected. A detailed outline of the experimental design is given in table 3.4. Three loops in six pigs were immunized with 125 μg F4 or 125 μg F4 plus 500 μg CpG ODN 2007 (sequence TCG TCG TTG TCG TTT TGT CGT T) per loop. Two loops per animal were treated with PBSA. Two of the pigs were $F4R^{neg}$, and the remaining four pigs were $F4R^{low}$ to $F4R^{mod}$.

The mean values of 15 F4-specific IgA-ASC (min. 1 – max. 116) and 20 IgG-ASC/ 5 x 10^6 cells (min. 1 – max. 251) were found in F4-immunized loops (Figure 3.10 (A)-(F)). In contrast mean values of 10 F4-specific IgA-ASC (min. 1- max. 53) and 17 IgG-ASC/ 5 x 10^6 cells (min. 1 – max. 333) were found in F4-CpG immunized loops (Figure 3.10 (A)-(F)). Interestingly, in $F4R^{neg}$ animals very low to moderate numbers of F4-specific ASCs were detected in immunized loops. Very low numbers of F4-specific IgA-ASC/ 5 x 10^6 cells (min. 1 – max. 17) and IgG-ASCs/ 5 x 10^6 cells (min. 1 – max. 4) were detected in control loops. Thus, no significant differences were seen in the number of F4-specific ASCs in loops immunized with crude F4 protein versus crude F4 protein plus CpG ODN.

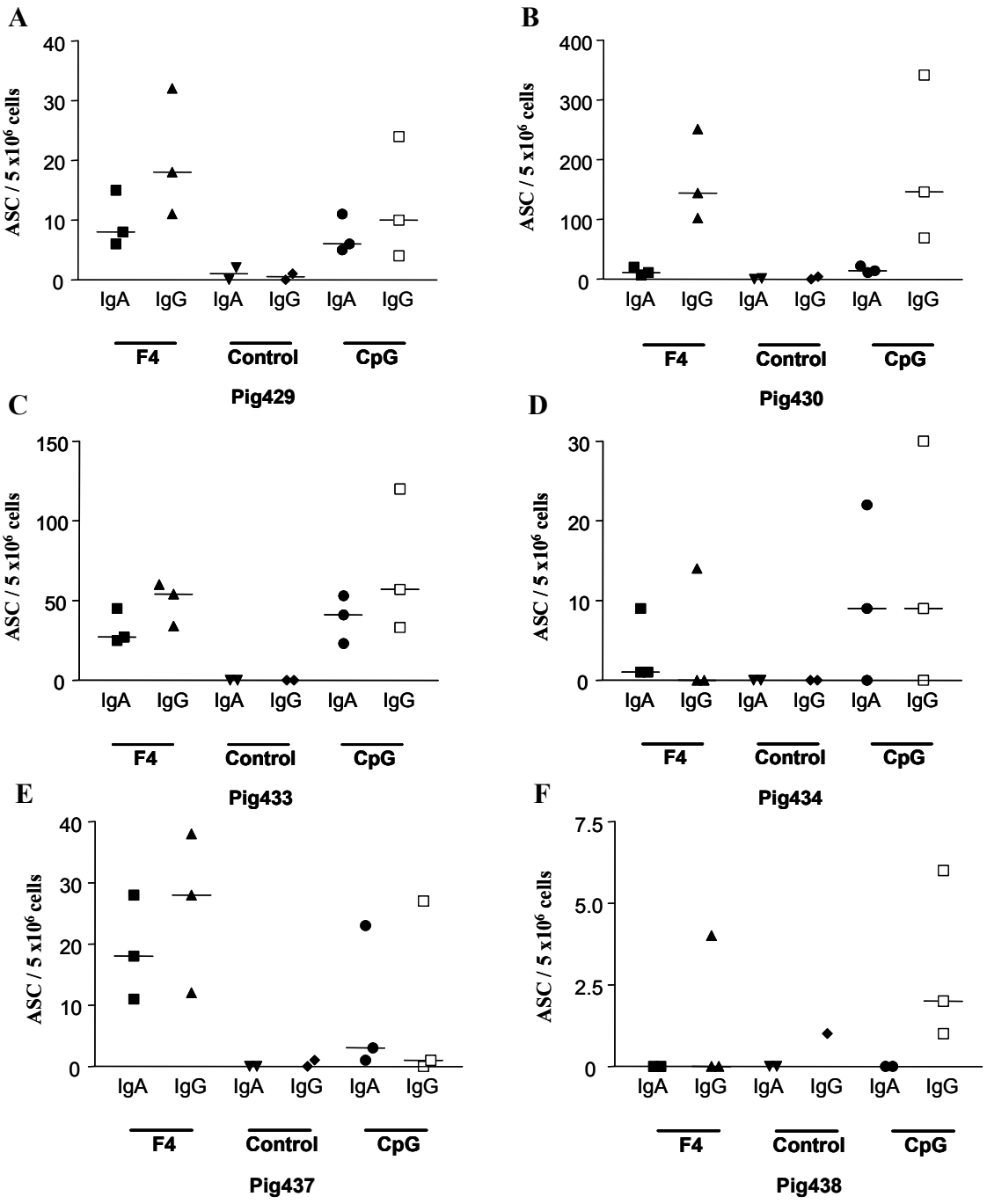


Figure 3.10 The number of F4-specific IgA- and IgG-ASC isolated from the PPs of gut-loops from pigs 429 (A), 430 (B), 433 (C), 434 (D), 437 (E) and 438 (F) are shown above. Control loops were administered 1 ml of PBSA, F4 immunized loops contained 125 μg crude F4 protein, and CpG immunized loops contained 125 μg of crude F4 protein + 500 μg CpG ODN 2007. Each IgA- and IgG-ASC value is the mean ± the standard deviation (SD) of a triplicate of wells each receiving 100 μl of 5 x 10^6 isolated follicular PP cells/ml. The F4R statuses of the pigs were 434^{neg}, 429^{++}, 430^{++}, and 433^{++}, 437^{-}, 438^{+}.

Table 3.4 Effect of CpG ODN 2007 on the induction of F4-specific antibody secreting cells in the gut. $F4R^{high\ (+++)}$, $F4R^{medium\ (++)}$, $F4R^{low\ (+)}$ $F4R^{neg\ (-)}$.

Exp 4	Pig	F4 µg/loop	F4-ASC in immunized loops		F4 + CpG µg/loop	F4-ASC in F4-CpG ODN immunized loops		F4-ASC in control loops	
			IgA	IgG		IgA	IgG	IgA	IgG
	429^{++}	125	15 ± 3 8 ± 3 6 ± 1	32 ± 5 11 ± 3 18 ± 5	125+500	6 ± 3 11± 2 5 ± 1	10 ± 2 24± 6 4 ± 1	2 ± 1 0 ± 0	1 ± 1 0 ± 0
	430^{++}	125	7 ± 1 20 ± 3 11 ± 2	144 ± 6 251 ± 19 102 ± 6	125+500	22 ± 2 11 ± 4 13 ± 1	332 ± 16 146 ± 21 69 ± 11	1 ± 1 0 ± 0	4 ± 0 0 ± 0
	433^{++}	125	25 ± 7 27 ± 9 45 ± 5	60 ± 9 54 ± 8 34 ± 8	125+500	23 ± 9 41 ± 7 53 ± 5	120 ± 23 33 ± 3 57 ± 12	0 ± 0 0 ± 0	0 ± 0 0 ± 0
	434^{-}	125	1 ± 1 1 ± 1 9 ± 2	14 ± 8 0 ± 0 0± 0	125+500	22 ± 1 0 ± 0 9 ± 2	30 ± 3 9 ± 3 0 ± 0	0 ± 0 0 ± 0	0 ± 0 0 ± 0
	437^{-}	125	28 ± 2 18 ± 4 11 ± 0	38 ± 2 12 ± 4 28 ± 3	125+500	23 ± 3 1 ± 1 3 ± 3	27 ± 6 1 ± 0 0 ± 0	0 ± 0 0 ± 0	0 ± 0 1 ± 1
	438^{+}	125	0 ± 0 0 ± 0	4 ± 3 0 ± 0 0 ± 0	125+500	N/A 0 ± 0 0 ± 0	6 ± 1 1 ± 1 2 ± 1	0 ± 0 0 ± 0	0 ± 0 *

Footnote: * - only one control loop.

3.4.9 The adjuvant pBD-1 did not enhance the local F4-specific immune response in loops when coadminstered with crude F4 protein

To test the effect of the pBD-1, three loops/pig in seven pigs were immunized with either 125 μg F4/loop or a combination of 125 μg F4/loop + 500 μg pBD-1/loop. Two control loops were injected with PBSA. Two of the pigs were $F4R^{neg}$, four were $F4R^{low\text{-}mod}$ and one was $F4R^{high}$. A moderate dose of the F4 (125 μg F4/loop) was chosen to be co administered with pBD-1 so that the effect of adjuvant could be detected. A detailed outline of the experimental design is given in table 3.5. The mean values of 15 F4-specific IgA-ASC/ 5 x 10^6 cells (min. 1 to max 116) and 20 IgG-ASC/ 5 x 10^6 cells (min. 1 to max. 251) were found in F4-immunized loops and 17 F4-specific IgA-ASC (min. 1 to max 69) and 22 IgG-ASC/ 5 x 10^6 cells (min. 1 to max. 155) were found in F4-pBD-1 immunized loops (Figures 3.11 and 3.12). $F4R^{neg}$ animals again displayed low to moderate numbers of F4-specific ASCs. Thus, no significant differences were seen in the number of F4-specific ASCs in loops immunized with crude F4 protein versus crude F4 protein plus pBD-1.

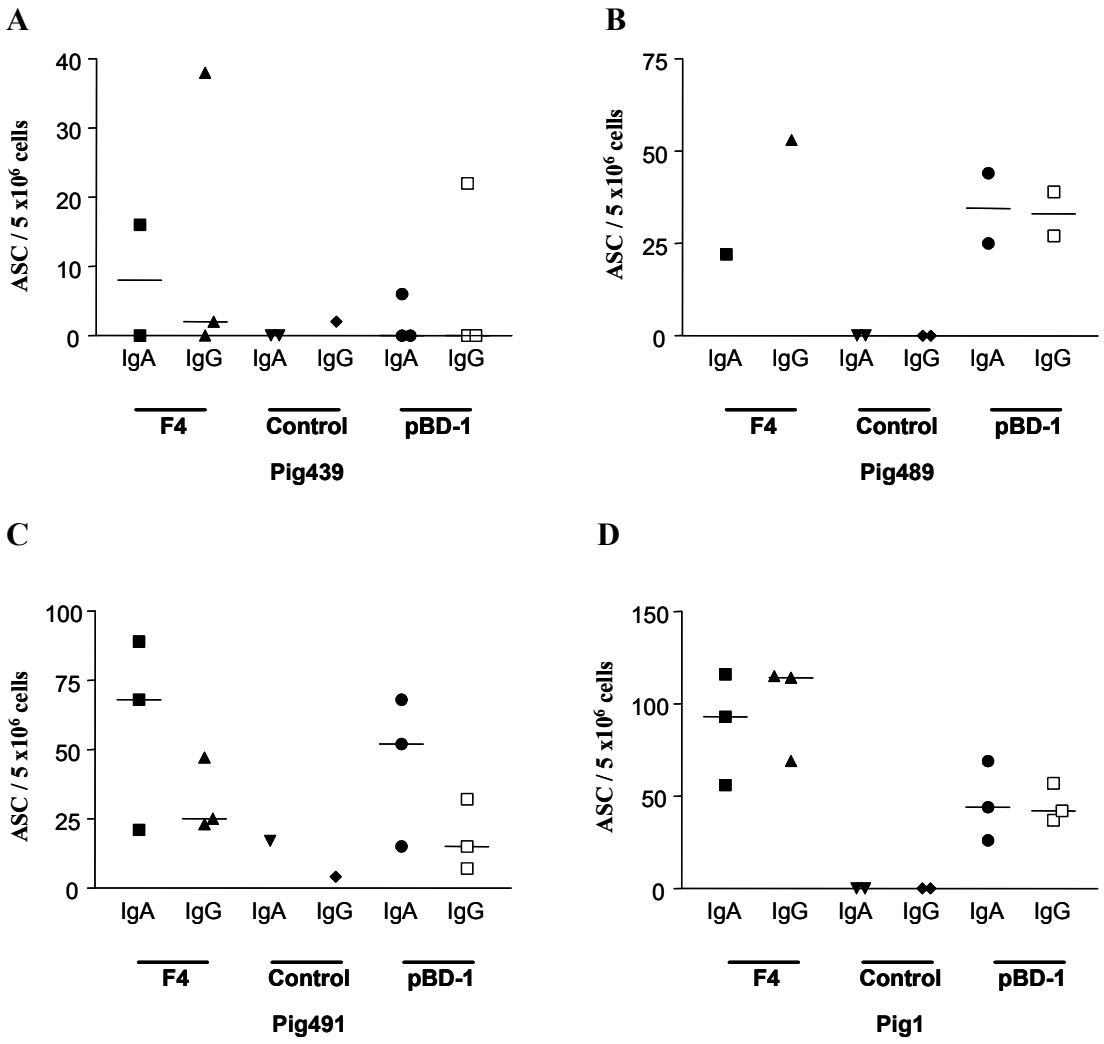


Figure 3.11 The number of F4-specific IgA- and IgG-ASC isolated from the PPs of gut loops from pigs 439 (A), 489 (B), 491 (C), and 1 (D) are shown above. Control loops were administered 1 ml of PBSA, F4 immunized loops contained 125 μg crude F4 protein, and pBD-1 immunized loops contained 125 μg of crude F4 protein + 250 μg porcine β defensin-1. Each IgA- and IgG-ASC value is the mean ± the standard deviation (SD) of a triplicate of wells each receiving 100 μl of 5 x 10^6 isolated follicular PP cells/ml. The F4R statuses of the pigs were 439^{neg}, 489^{++}, 491^{+++}, and 1^{+}.

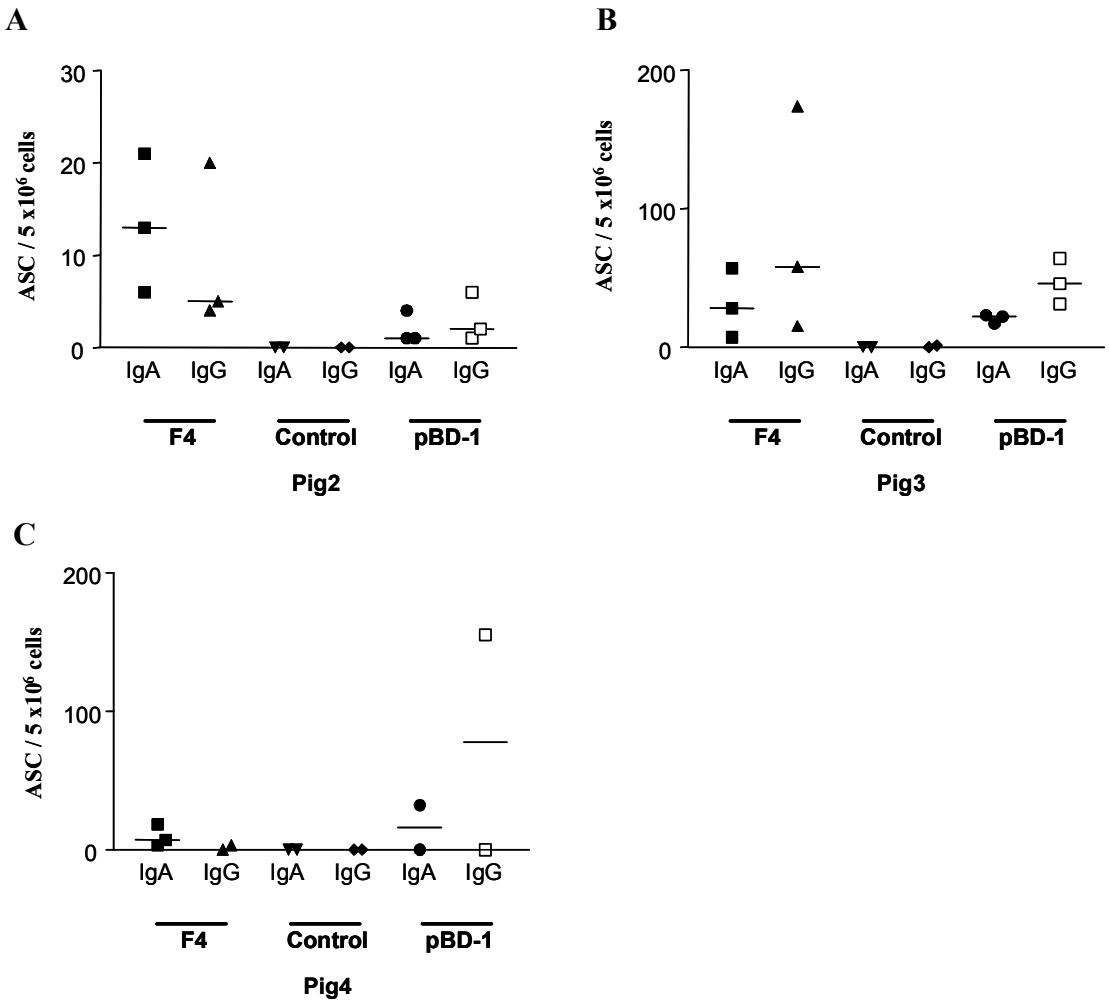


Figure 3.12 The number of F4-specific IgA- and IgG-ASC isolated from the PPs of gut loops from pigs 2 (A), 3 (B), 4 (C) are shown above. Control loops were administered 1 ml of PBSA, F4 immunized loops contained 125 μg crude F4 protein, and pBD-1 immunized loops contained 125 μg of crude F4 protein + 250 μg porcine β defensin-1. Each IgA- and IgG-ASC value is the mean ± the standard deviation (SD) of a triplicate of wells each receiving 100 μl of 5 x 10^6 isolated follicular PP cells/ml. The F4R statuses of the pigs were 2^{neg}, 3^{+}, and 4^{++}.

Table 3.5 Effect of pBD-1 on the induction of F4-specific antibody secreting cells in the gut. $F4R^{high\ (+++)}$, $F4R^{medium\ (++)}$, $F4R^{low\ (+)}$ $F4R^{neg\ (-)}$.

Exp	Pig	F4 µg/loop	F4-ASC in F4-immunized loops		F4 + pBD-1 µg/loop	F4-ASC in F4-pBD-1 immunized loops		F4-ASC in control loops	
5			**IgA**	**IgG**		**IgA**	**IgG**	**IgA**	**IgG**
	439 $^{-}$	125	16 ± 2 0 ± 0 6 ± 1	38 ± 6 2 ± 2 0 ± 0	125+250	6 ± 1 0 ± 0 0 ± 0	22 ± 3 0 ± 0 0 ± 0	0 ± 0 0 ± 0	2 ±0 *
	489 $^{++}$	125	22 ± 4 ***	53 ± 4 ***	125+250	44 ± 2 25 ±7 **	39 ± 12 27 ± 4 **	0 ± 0 0 ± 0	0 ±0 *
	491^{+++}	125	68 ± 4 89 ± 21 52 ± 9	25 ± 2 47 ± 10 23 ± 5	125+250	68 ± 4 52 ± 13 15 ± 6	32 ± 5 15 ± 2 7 ± 1	17 ±5*	4 ±0*
	1 $^{+}$	125	116 ± 16 93 ± 9 56 ± 2	115 ± 14 114 ± 10 69 ± 6	125+250	44 ± 7 69 ± 17 26 ± 6	42 ± 14 57 ± 14 37 ± 4	0 ± 0 0 ± 0	0 ± 0 0 ± 0
	2 $^{-}$	125	21 ± 4 13 ± 3 6 ± 3	20 ± 6 5 ± 2 4 ± 1	125+250	4 ± 3 1 ± 1 1 ± 1	6 ± 2 2 ± 2 1 ± 0	0 ± 0 0 ± 0	0 ± 0 0 ± 0
	3 $^{+}$	125	28 ± 9 57 ± 19 7 ± 1	58 ± 24 174 ± 21 15 ± 3	125+250	23 ± 6 22 ± 8 17 ± 5	31 ± 4 64 ± 4 46 ± 6	0 ± 0 0 ± 0	1 ± 0 0 ± 0
	4 $^{++}$	125	3 ± 1 18 ± 4 7 ± 3	3 ± 3 0 ± 0 N/A	125+250	0 ± 0 32 ± 6 N/A	0 ± 0 155 ± 10 N/A	0 ± 0 0 ± 0	0 ± 0 0 ± 0

Footnote: * - only one control loop; N/A – not enough cells collected; ** - only one immunized loop was viable***

3.4.10 Importance of the F4R for the local immune response

To analyze the role of the F4R for the eliciting of the F4-specific immune response in our gut-loops we examined all loops from all pigs except those deemed secondary responders (cohorts 429, 430, and 491). Doses of 0, 50, 125, 250, and 500 μg/ml were administered to loops and the expression level of the F4R as determined by the IVVA on the F4-specific immune response was measured by taking the log of the F4-specific IgA or IgG ASCs isolated from loop PPs.

There was a significant effect of F4R expression upon the number of IgA-ASCs ($P< 0.0001$; r^2 value= 41%) and IgG-ASC ($P < 0.0001$; r^2 value= 0.36) (Figures 3.13 and 3.14) (also see appendix for a more detailed analysis). Interestingly when the non-immunized loops from all cohorts were removed from the data set a dose effect was not observed for neither the F4-specific IgA-ASCs (P=0.13) nor IgG-ASCs (P=0.87) responses (Figures 3.16 and 3.17) (also see appendix for a comprehensive analysis of loop data). Furthermore, increased doses of F4 Ag were not always associated with an upward shift in the dose response curve see Figures 3.16 and 3.17. Therefore, the statistical effect of dose was due to administering the crude F4 protein rather than increasing its dose. Thus, these results demonstrate that the level of F4R expression and the event of immunization were crucial for the induction of local immunity in the small intestine of pigs.

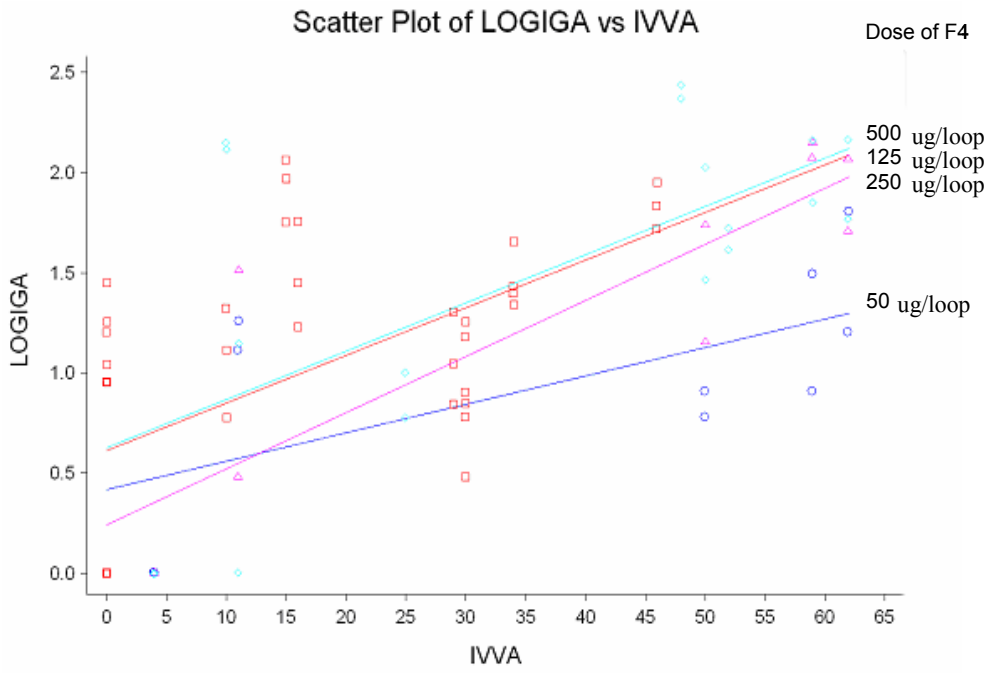


Figure 3.13 Using only the data from loops administered various doses of crude F4 protein, only the expression level of the F4R ($P < 0.0001$) was significantly associated with the log of the IgA-ASC immune response. R-squared = 41%. Dose however was not associated with the log of the IgA-ASC immune response ($P = 0.13$). (50 µg ○ , 125 µg □ ,250 µg △ , and 500 µg ◇).

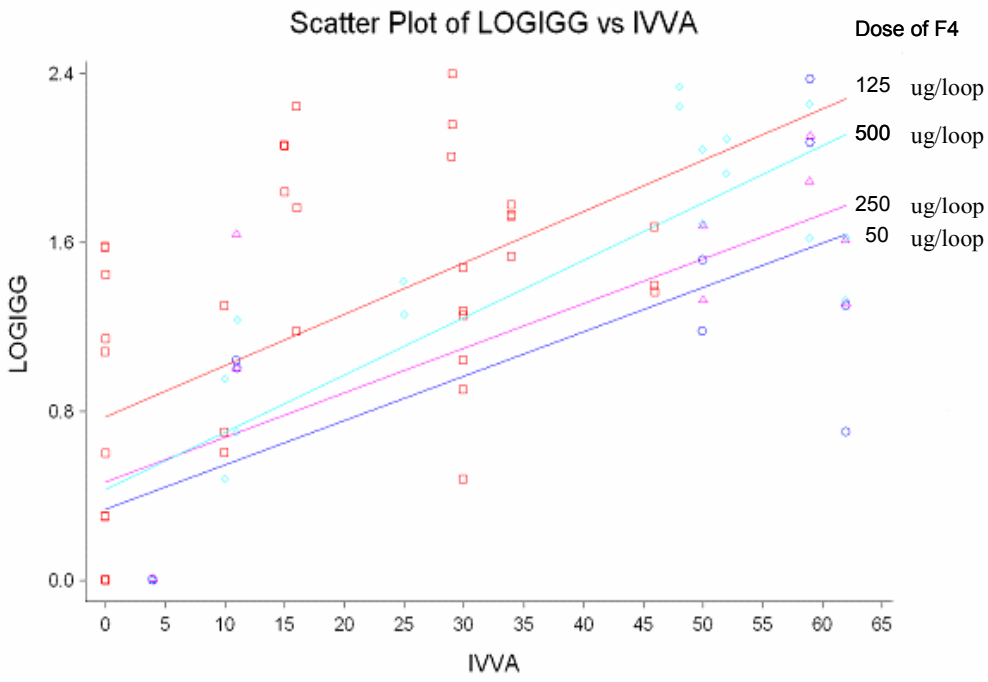


Figure 3.14 Using only the data from loops administered various doses of crude F4 protein, only the expression level of the F4R ($P < 0.0001$) was significantly associated with the log of the IgG-ASC immune response. R-squared = 36%. Dose however was not associated with the log of the IgG-ASC immune response ($P = 0.87$ (50 µg ○ , 125 µg □ , 250 µg △ , and 500 µg ◇).

3.4.11 F4R^{pos} and F4R^{neg} pigs grouped according to the number of F4-specific ASCs in immunized loops versus F4-specific ASCs in non-immunized loops.

F4R^{pos} pigs could be grouped according to the number of F4-specific ASCs in immunized loops versus F4-specific ASCs in non-immunized loops (Table 3.6). Eleven F4R^{pos} pigs had expected outcomes of vaccination as they displayed ≥ 10 F4-specific ASCs in immunized loops and no F4-specific ASCs in control loops. Two F4R^{pos} pigs had < 10 F4-specific ASCs in immunized loops and no F4-specific ASCs in control loops. The four remaining F4R^{pos} pigs had F4-specific ASCs in immunized loops and F4-specific ASCs in control loops however two of these pigs had > 10 F4-specific ASCs in their control loops indicating that they may have been preexposed to ETEC F4 (2^{o} responders) prior to immunization. Five F4R^{neg} pigs were also grouped according to their response to F4-immunization (Table 3.7). Three of these pigs had > 10 F4-specific ASCs in immunized loops and no F4-specific ASCs in their control loops, which indicates that F4R^{neg} pigs indeed respond to vaccination. Only one F4R^{neg} pig had no F4-specific ASC in both immunized and non-immunized loops.

Table 3.6. F4R^{pos} pigs were grouped based upon the number of F4-specific ASCs in immunized loops versus F4-specific ASCs in non-immunized loops.

F4-ASC in immunized loops	F4-ASC in control loops	Number of animals
≥ 10	**0**	**11**
< 10	**0**	**2**
≥ 10	**< 10**	**2**
> 10	**> 10**	**2**

Table 3.7. $F4R^{neg}$ pigs were grouped based upon the number of F4-specific ASCs in immunized loops versus F4-specific ASCs in non-immunized loops.

F4-ASC in immunized loops	F4-ASC in control loops	Number of animals
≥ 10	0	3
< 10	0	2
≥ 10	< 10	0
> 10	> 10	0

3.4.12 F4-specific serum antibody response following intestinal immunization

Serum samples were taken from all our pigs on the day of surgery and on day twenty-four and analyzed for the presence of F4-specific IgG immunoglobulin's. Out of twenty-two pigs analyzed only five showed noteworthy increases in serum F4-specific titers after F4-immunization into our gut-loops (Table 3.8). This indicates that perhaps F4-immunization into our loops was not effective in eliciting systemic F4-specific IgG.

Table **3.8.** F4-specific serum antibody titers at the day of gut-loop surgery and at the time of tissue collection.

Pig	Day 0	Day 24
180	68	46
181	795	480
182	63	51.5
183	52	103
217	113	323
218	58	61
220	113	39.5
221	51	16
222	50	39
429	95	41
430	0	12
433	35	36
434	17	39
437	104	82
438	145	63
439	159	64
489	9	46
491	15	20
1	10	60
2	47	14
3	110	34
4	75	28

3.4.13 The effect of F4-specific serum antibody on the F4-specific Ig-ASC response in the loops

Interestingly, some pigs had higher F4-specific IgG antibody levels in their serum at day 0 (Table 3.8). This could be the result of prior exposure to ETEC F4 or perhaps passive transfer of maternal antibodies (mAbs) from the sow. Therefore, we decided to examine if F4-specific serum antibodies interfered with our gut-loop immunization by assessing the factors dose, the expression level of the F4R, and the F4-specific IgG titer at day 0. These variables were assessed in regression models with the log-transformed IgA- and IgG-ASCs in immunized loops as the outcome. Furthermore, pigs that we thought were 2^{o} responders (220, 491, 430 and 431) were excluded from this analysis. Our results indicated that there was a statistical effect of the F4-specific serum IgG to inhibit the F4-specific IgA-ASC response in our gut-loops (P=0.004; for a detailed analysis see appendix). However this was not the case for IgG (P=0.08; for a detailed analysis see appendix).

Thus, F4-specific IgG in the serum affected intestinal immunization with the native F4 protein in our loops. Further investigation is required to understand the mechanism of inhibition.

3.5 Discussion

Protective vaccination against *E. coli* depends on the induction of local immune responses in the small intestine. Here, we show that vaccination with as little as 50 μg of purified F4/ loop resulted in the induction of local mucosal immune responses in immunized loops, but not in non-immunized control loops. Furthermore, we demonstrate that purified F4 induced weak immune responses in some $F4R^{neg}$ animals. Interestingly, the induction of local immune responses was dependent on the level of F4R-expression within each loop: Whereas $F4R^{neg\text{-}low}$ pigs mounted only weak immune responses in immunized loops, $F4R^{mod\text{-}high}$ pigs mounted consistently higher F4-specific Ig ASC immune responses and displayed the most significant response to dose. Thus, expression of the F4R and binding of its ligand, the F4 protein, seemed critical for the induction of intestinal F4-specific immunity in our gut-loops.

Uptake of F4 antigen across the mucosal surface can be mediated by either M cells that are located in the follicle-associated epithelium above PPs or by dendritic cells DCs that sample the antigen from the gut lumen. Both mechanisms are non-specific and theoretically could take place in both $F4R^{pos}$ and $F4R^{neg}$ animals. However, our results suggest that uptake of the F4 is more efficient in $F4R^{mod\ to\ high}$ pigs as these animals mounted stronger immune responses against the F4. Thus, binding of the F4 to the F4R must result in an augmented uptake across the mucosal lining.

M cells are specialized epithelial cells that preferably take up particulate antigen and it is most likely that binding of the F4 to the F4R would not alter this process. However, it is not known if porcine M cells express the F4R. In contrast, binding of the F4 to the F4R could significantly increase uptake by DCs. For example, the closer

proximity of the bound antigen could allow for a more efficient sampling by DCs as dendrites would be able to reach more of the antigen. Alternatively, binding to the F4R could also result in internalization of the F4 by epithelial cells, which would then pass the F4 onto DCs that are located underneath the epithelial layer. Both mechanisms favor the role of DCs and indicate that uptake and subsequent immune presentation by DCs represents the major pathway for the induction of F4-specific immunity in the intestine.

Support for this hypothesis comes from the present data, which shows that $F4R^{neg}$ and $F4R^{low}$ pigs only mount weak responses against F4 and from observations made by Van den Broeck et al., 1999 who first showed that immune responses were not detectable in orally immunized $F4R^{neg}$ pigs. However, the same authors also reported that $F4R^{neg}$ pigs had secondary responses following systemic booster immunization with F4, which indicated that priming had taken place in these animals [12]. Thus, we conclude that immunity against the F4 can be induced in $F4R^{neg}$ pigs. However, responses are very weak and often below the level of detection, which would explain the non-responsiveness to the primary immunization described by Van den Broeck et al., 1999. In the present study, we have used a gut-loop model, which allows a more controlled antigen delivery and much more detailed analysis of local immunity in the small intestine. Administration of F4 into surgically ligated loops and subsequent isolation of F4-specific cells specifically from these sites of immunization could provide a much higher level of sensitivity for detecting even weak immune responses and therefore could explain why in some $F4R^{neg}$ animals F4-specific immune responses were found even after a primary immunization. However, more studies are required for a comprehensive understanding of the induction of mucosal immunity in $F4R^{neg}$ pigs.

DCs are found along the mucosal surfaces of the intestine in several species including pigs [21]. Uptake of bacterial antigens from the intestinal lumen was first described in mice by Rescigno et al., (2001) and further explored by others [22]. Several subpopulations of DCs have been described in mice that can take up soluble antigen from the gut-lumen. These cells subsequently migrate towards the follicular zones and follicles within PPs or mesenteric LNs, where they can present the antigen to the immune system [23]. Although the existence of these DC subpopulations has not been confirmed in pigs, it is likely that DCs with similar functions exist in swine. Currently however, their exact location and proximity to the PP needs to be determined.

Furthermore, evidence for the importance of DCs in the induction of F4-specific immune responses in the small intestine comes from studies in our lab, which demonstrated that F4-specific immune responses can be induced in interspaces, which are loops that lack PPs as major inductive sites [24]. Thus, DCs play an important role in uptake of the F4 Ag and induction of F4-specific immune responses in the gut. Since F4-specific ASCs were found in both the PP and the mesenteric LN (data not shown), one can assume that DCs after uptake of the F4 Ag migrate towards the PP or directly towards the mesenteric LN to prime effector cells within the interfollicular and follicular regions respectively. Currently experiments in our lab are underway to analyze sampling of the F4 Ag by intestinal DCs, and ways to exploit the F4 Ag-F4R interaction to improve vaccines.

Interestingly, in the present study we tried to improve upon the F4 Ags immunogenicity by using adjuvants i.e. CpG ODN, to enhance the number of F4-specific ASCs within immunized loops. In the past, several groups using a variety of species

including pigs have demonstrated the adjuvanting activity of CpG ODN. For example, systemic administration of CpG ODN to pigs enhanced immune responses against F4 and the *Actinobacillus pleuropneumoniae* outer membrane protein (OmlA) [8, 25]. However, CpG ODN was only administered systemically in these studies. The potential of CpG ODN to function as mucosal adjuvant was reviewed by McCluskie et al., (2000) [26]. In our pig gut-loops, we chose ODN 2007, a member of the B-class ODN, which are known to enhance B cell responses and therefore are considered the most effective adjuvanting type of CpG ODN. In addition, the immunostimulatory activity of ODN 2007 had been previously demonstrated in pigs [27, 28]. Further, the dose of 500 µg / loop derived from gut-loop studies in sheep, demonstrated that 500 µg CpG ODN / loop was most effective in adjuvanting immune responses against intestinally administered porcine serum albumin [29]. In contrast to these observations, however, combination with CpG ODN 2007 did not enhance the local immune response against F4 in our present study. Several factors such as insufficient CpG ODN dosing, inadequate formulation, and the time point of assessment could represent possible reasons for this failure. Clearly, more studies with larger number of animals are required to identify optimal efficacy of CpG ODN for intestinal immunization with F4.

Similarly, this was also the case for the pBD-1, which was included in the present study because of its immunomodulatory activity *in vitro* and *in vivo* [30]. Defensins have been described as potent immunomodulators that can modify immune responses against bacterial, viral and tumor antigens. However, very little information is currently available about stability and formulation, as well as optimal dosing and administration of these

compounds. Thus, further experiments are necessary to identify the potential of these immunomodulators for future vaccines.

Vaccination against ETEC in both human and animals is complicated by a number of factors including interference with maternally-derived antibodies. Our results indicated that higher F4-specific antibody levels at the time of immunization interfered with the induction of mucosal immune responses in immunized loops. This is a well established concept, and in fact represents one of the biggest challenges in vaccinating the newborn infant or animal. Thus, more effective vaccines are required that can overcome this interference and indeed the pig provides a very useful model for understanding mucosal immunity against ETEC. The pig model could also be of relevance for enhancing our understanding of *E.coli* infections causing diarrhea in other species. For example, receptors for colony forming antigen (CFA) homologous to the F4R are found in humans making our results relevant for important diseases such as Traveler's diarrhea. Moreover, our results demonstrate that these antigen-specific receptors in fact play an essential role for the induction of immunity against ETEC and demonstrate that uptake via M cells is not necessary for the induction of local immunity. This is an important finding since it is commonly believed that M cell uptake and induction of immunity within the Peyer's patch are essential for the induction of mucosal immunity in the small intestine. Furthermore, these findings also have implications for the development of novel vaccine strategies against ETEC in both animals and humans as it may be possible to target antigen-specific receptors on mucosal epithelial cells to increase vaccine efficacy. Nevertheless, more studies are necessary to understand the

induction of mucosal immunity against *E. coli* and develop effective vaccines for both human and animals.

3.6 Acknowledgements

We would like to thank Julia Whale, Rachelle Buchanan, and Janet Foley for their technical assistance, and Dr. Kuldip Mirakhur and the VIDO Animal Care Staff for performing surgeries, treatment and housing of animals. We thank Merial Limited, Duluth, Georgia for CpG ODN 2007. Funding was provided by the Saskatchewan Agriculture Development Fund and the Canadian Network for Bacterial Pathogens of Swine. Manuscript published with permission of the Director of the Vaccine & Infectious Disease Organization as article # 428.

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5. DISCUSSION

Our study demonstrated that a local F4-specific immune response was seen in the gut-loops of pigs immunized with the F4 Ag. The strength of the F4-specific immune response depended upon the expression level of the F4R. The effect of dose on the F4-specific immune response seen in the loops was statistically insignificant when the dose of 0 (saline control) was removed from the analysis. The level of serum antibodies found in the weaned pig had a negative effect on the magnitude of the F4-specific immune response in the loops. Unfortunately, the F4-specific immune response in the loops was not enhanced using the adjuvants CpG ODN or the defensin pBD-1.

The idea that the F4R is required to elicit an F4-specific immune response against the F4 Ag is not new. For over 30 years it has been known that $F4R^{neg}$ pigs as apposed to $F4R^{pos}$ pigs, would not get sick when infected with ETEC F4 thus establishing a genetic basis regarding the resistance and susceptibility for this disease [23]. Moreover, we know that proteins like fimbria, lectins, and viral hemagglutinins, which have cognate receptors on animal membranes, elicit much more potent immune responses when delivered orally to animals than proteins that do not possess similar binding activities [141]. Van den Broeck et al., 1999 reconfirmed this by vaccinating pigs with crude F4 protein demonstrating that this lectin like protein elicits F4-specific immunity in the small intestine.

Similarly our group using intestinal loops in pigs also tested the immunogenicity of the crude F4 fimbrial protein and found that it was immunogenic in our gut-loops.

However, in order to demonstrate that the crude F4 protein was responsible for the F4-specific immune response, we isolated the F4 on an SDS PAGE gel and verified its presence by Western blot with an F4-specific rabbit polyclonal antibody. Moreover, we showed that the crude F4 protein is specific for the F4R through inhibiting the adherence of ETEC F4 in a series of modified IVVAs. For example, we pre-incubated ETEC F4 with crude F4 fimbrial protein, mMAb, or rPAb, prior to co-incubation with $F4R^{pos}$ villi. All of these inhibitors worked to block the binding of ETEC F4 to $F4R^{pos}$ villi in a dose dependant manner. Thus our inhibition assays demonstrated that our crude F4 fimbrial protein is specific for the F4R in the small intestine of pigs.

Interestingly, we found that an electroeluted version of the F4 protein did not elicit F4-specific Ig-ASCs in our first gut-loop experiment. This was likely due to its failure to reassemble into a form that was capable of binding the F4R, since the denaturing gel used in the purification process removed the minor subunits which are required in the bacteria for correct assembly of the nascent fimbriae [20]. However since OVA did not elicit OVA-specific Ig-ASCs in OVA immunized loops, the possibility exists that the negative results we obtained were due to technical problems. Therefore due to the high cost of each gut-loop trial it was decided that we would use the crude form of the F4 fimbrial protein for all future experiments. Indeed in subsequent trials investigating the crude F4 Ag, it elicited F4-specific immune responses in the loops of both $F4R^{pos}$ and $F4R^{neg}$ pigs.

The detection of F4-specific immune responses in $F4R^{neg}$ pigs after immunization with solubulized F4 protein in our gut-loops was unexpected. This is because Van den Broeck et al., 1999 after orally administering crude F4 protein to weaned pigs indicated

that no F4-specific immune responses were seen in $F4R^{neg}$ pigs but only a priming was detected. Thus our model in contrast to oral vaccination, allowed for the delivery of relatively large amounts of F4 Ag to a confined space (the loop) permitting us to immunize and look for F4-specific immune responses at the site of immunization. Thus our gut-loops permitted us to detect the on average of < 10 F4-specific ASC / 5 x 10^6 cells typically present in the PPs of $F4R^{neg}$ pigs.

On the other hand, nominal F4-specific immune responses like the ones detected in the gut-loops of our $F4R^{neg}$ pigs likely go unnoticed in oral immunization protocols because of the methods used to detect them. For example, fecal samples are typically collected and probed for F4-specific SIgA. However this method likely is not sensitive enough to detect the low numbers of F4-specific SIgA present in the feces of these pigs. Another method used to detect the F4-specific intestinal immune response to F4 oral immunization is the isolation of PBMCs from the blood. However, given the low number of F4-specific ASCs typically found in $F4R^{neg}$ pigs there may not be enough F4-specific monomorphonuclear cells circulating in the blood to be detected [43]. Thus, our gut-loop immunization protocol allowed us to detect the nominal F4-specific immune responses seen in $F4R^{neg}$ pigs because we isolated F4-specific Ig-ASCs directly from PPs of loops that were immunized with the F4 protein.

Interestingly, our gut-loop immunization did not elicit strong F4-specific IgG immune responses in the systemic immune compartment of $F4R^{neg}$ or $F4R^{pos}$ pigs. Evidence for this could be inferred from the fact that we were unable to isolate F4-specific IgG-ASCs from the mesenteric lymph nodes in a majority of the pigs perhaps due to their frequency being to low. Moreover weak F4-specific immune responses in the

systemic immune compartment could have been masked by waning levels of maternal antibodies. Therefore until we modify our ELISA to detect either sow (IgG2A) or piglet IgG (IgG1) in the serum we can not conclude if F4-loop immunization elicits F4-specific immune responses in the serum.

Interestingly, a statistical analysis of the pre-immune serum IgG revealed that it was inhibitory upon the F4-specific IgA-ASC response seen in the loops after immunization with the F4. A potential reason for this is that maternal immunoglobulins still present in the mucus layer of the small intestine of the weaned pig could be inhibiting the binding of the F4 Ag to the F4R preventing uptake and subsequent induction of the F4-specific immune response. Thus, it appears that passive immunity transferred by the sow to the piglet during the suckling period can influence the outcome of F4-immunization in our loops. Therefore in future pig gut-loop trials we can prescreen sows for their levels of F4-specific IgG and use only piglets that are born to F4 seronegative mothers, and or we can prescreen the serum and feces of piglets for their levels of the F4-specific Ig, eliminating those cohorts with high levels of F4-specific antibodies.

In the current study for statistical purposes we eliminated pigs we deemed secondary responders i.e. pigs 429, 430, and 491. The removal of these pigs from the analysis allowed us to use only those animals that would elicit primary immune responses and not anamnestic ones after F4-loop immunization. Interestingly, their removal did not affect the outcome of the analysis regarding the effect of dose on the F4-specific immune response, or the relationship observed between the expression level of the F4R and the F4-specific immune response. Conversely another group of pigs, the $F4R^{neg}$ pigs, were

important cohorts for our statistical analysis because they provided us with data that reflected how the presence of the F4R was integral for eliciting F4-specific immune responses in the loops. Moreover our study revealed these pigs elicited F4-specific immune responses after immunization with crude F4 protein, thus they were included in our analysis regarding the expression level of the F4R and the F4-specific immune response.

Some question however existed as to whether a mucus form of the F4R could be acting as a sink, sequestering the F4 Ag in the loops of $F4R^{neg}$ pigs thereby inhibiting their abilty to elicit F4-specific immune responses. However since no F4R exists on the enterocytes of $F4R^{neg}$ pigs, the source of the F4R in mucus is thereby removed eliminating this as a reason for the hyporesponsiveness seen in $F4R^{neg}$ pigs after immunization with the F4 Ag [24]. Therefore an F4R independent mechanism regarding the uptake of the F4 Ag and induction of the F4-specific immune response in $F4R^{neg}$ pigs could be explained through an M cell mediated pathway. Thus once Ag is taken up in the PPs of these pigs, DCs located in the dome region of the follicle associated epithelium (FAE) capture the F4 Ag and subsequently migrate to the interfollicular region, or the mesenteric lymph nodes, where they can induce an immune response [136].

In contrast, the $F4R^{mod\text{-}high}$ pigs likely utilize the F4R and PP for a synergistic effect regarding F4 Ag uptake and induction of the F4-specific immune response. This is evident as data from our gut-loop experiments demonstrated that only loops with moderate to high levels of the F4R + PPs but not loops with only PPs, elicited high numbers of F4-specific ASCs. Moreover, Salles et al. 2005 showed that interspaces (devoid of PPs) in $F4R^{mod\text{-}high}$ pigs but not $F4R^{low\text{-}neg}$ pigs, elicited high numbers of F4-

specific ASCs after immunization with the crude F4 protein. Thus, it appears that the F4R is critical for the induction of F4-specific immunity; however, its exact role as a mediator of this process is still unknown. Therefore, we can only now speculate as to its mechanism.

A possible mechanism of induction of F4-specific immunity could be that the F4R on enterocytes binds the F4 fimbriae, (26 Kd), and participates in a clatherin mediated pathway of endocytosis of the F4 Ag, analogous to the uptake of Apo B-100 (30 Kd) by the low density lipoprotein receptor located in the liver [142]. This method of uptake could allow intestinal epithelial cells to participate as Ag presenting cells that pass the F4 Ag to DCs in the lamina propria, inducing an F4-specific immune response. However since porcine enterocytes do not possess MHC class II molecules, this mechanism may not be an efficient way to induce F4-specific immunity [143].

Another scenario involving the F4R would be if iDCs sample tethered F4 protein from intestinal enterocytes. Evidence to support DC sampling of Ag in the intestinal lumen comes from studies done in mice. Rescigno et al. (2001) found that DCs could extend their dendrites through the tight junctions of intestinal epithelial cells to sample bacteria present in the lumen in a process dependant upon their production of the tight junction proteins occludin, claudin 1, and zonula occludens 1 [55]. Furthermore, Niess et al. (2005) recently described a subset of murine DCs expressing the chemokine receptor CX_3CR1 that form transepithelial dendrites in response to the chemokine CX_3CL1/fractalkine produced by intestinal epithelial cells. However, CX_3CL1 is expressed at its highest levels in the ileum, and these CX3CR1 expressing DCs are believed to be important for the clearance of entero-invasive pathogens such as

Salmonella typhimurium [56]. Thus, it is possible that similar subsets of DCs exist that are capable of sampling F4 Ag from the lumen of $F4R^{pos}$ pigs and presenting F4 Ag to lymphocytes in the PPs and or mesenteric lymph nodes. This makes the F4R an integral component in DC sampling by functioning to make F4 Ag uptake more efficient.

This was evident in our loops as more F4R in a loop typically translated into higher numbers of F4-specific ASCs after F4 immunization. Therefore increasing the dose should have resulted in greater F4 binding to the F4R leading to more Ag sampling resulting in higher F4-specific immune responses. Interestingly, our statistical analysis revealed that after taking into account the expression level of the F4R, and dose, the event of immunization rather than the concentration of the F4 protein was important for eliciting an F4-specific immune response in the loops. Therefore, in order to enhance the F4-specific immune response in our gut-loops perhaps an adjuvant was needed.

To this end we proposed to enhance the local F4-specific immune response in our gut-loops by using the adjuvant CpG ODN. Unfortunately, CpG ODN did not increase the F4-specific immune response in our gut-loops. However, Mutwiri et al. (2002) using sheep gut-loops, demonstrated that CpG ODN coadministered with porcine serum albumin (PSA) enhanced the PSA-specific immune response in these loops. Furthermore they showed that CpG ODN was effective at 500μg when coinjected with PSA into the loops [89]. Therefore, we decided to use 500 μg in our pig gut-loops. Unfortunately, the class B CpG ODN 2007 used in our gut-loop experiment did not have an immunoenhancing effect. This could have been due to 500μg being an ineffectual dose, or the need for CpG ODN + F4 to be co delivered within some sort of delivery vehicle such as a liposome or Poly (DL-lactic-co-glycolic acid), shown by others to enhance the

immunostimulatory activity of CpG [144, 145]. Furthermore, kinetic studies may need to be carried on CpG ODN in our loops out so as to determine an optimum time point for harvesting our loops after its co-injection with the F4. Therefore, in order to potentially obtain an adjuvant effect from CpG ODN in future pig gut-loop experiments we may need to incorporate some or all of the measures noted above.

Similarly, pBD-1 was ineffective at enhancing the F4-specific immune response in the loops when co-administered with the F4 Ag. We expected pBD-1 to enhance the F4-specific immune response by 1) chemoattracting more iDCs to sample luminal F4 Ag and 2) promote iDC maturation, thereby increasing the number of mature DCs migrating to the PPs or mesenteric lymph nodes to present the F4 Ag more effeciently. However, it appeared that co-administration of the F4 + pBD-1 in our loops was ineffective at the dose tested. Therefore, like CpG ODN we may need to co-deliver the F4 and pBD-1 within a novel delivery vehicle so as to increase its supposedly short lifetime of activity *in vivo* i.e 4-6 hours [30]. The short life-time of pBD-1 *in vivo* could mean that it might be enhancing the F4-specific immune response shortly after immunization requiring us to harvest our loops at earlier time intervals, perhaps between 14-21 days post immunization in order to detect a response. Furthermore, we could also link the F4 to pBD-1 creating a fusion peptide. Thereby bringing the F4 and adjuvant closer together, creating a unique spatial and temporal effect regarding uptake of the F4 Ag and activation of iDCs. Thus, we cannot conclude that the adjuvants CpG ODN and pBD-1 are inefficacious to enhance F4-specific immunity in pigs, until further testing employing the methods above are tried.

Nevertheless, we currently have no adjuvant that can be administered orally with the crude F4 fimbrial protein that can elicit herd immunity against PWC within a group

of pigs. Thus, new vaccination strategies are needed that can elicit strong F4-specific immune responses in all pigs without the presence of the F4R. One strategy could involve the use of a viral vector containing the F4 Ag that could target and enter enterocytes independent of the F4R leading to copious amounts of F4 Ag expression and high F4-specific intestinal immune responses. Thus, novel strategies such as this to combat enteric infections caused by *E.coli* could be tested in the pig first, and if successful used in other species to prevent ETEC infection.

Specifically, consider the disease known as Traveler's diarrhea in humans, like PWC is caused by *E.coli* that use fimbriae known as colony forming Ags (CFAs) to facilitate adherence and colonization of the intestine [146]. It is unknown if the enterocyte receptor for the CFA is variably expressed across the human population like the F4R is in pigs. However if a similarity exists, then orally vaccinating humans with a CFA based vaccine to prevent Traveller's diarrhea may not be effective due to the inability to generate good immune responses in those individuals with low to moderate expression levels of the CFA receptor in their small intestine. Therefore ETEC will persist in the environment i.e in feces and contaminated water, maintaining infectivity. Thus the pig model is able to teach us some valuable lessons for developing vaccines against *E.coli* that can cause enteric infections in humans and other species.

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7. APPENDIX

7.1 STATISTICAL ANALYSIS

Analysis 1. All loops from 21 pigs were examined for the effect of dose (0, 50, 125, 250, & 500 ug/ml F4) and the expression level of the F4R as determined by the *in vitro* villous assay (IVVA) on the F4-specific immune response measured by taking the log of the F4-specific IgA antibody secreting cells (ASC) isolated from Peyers patches.

Result – Both dose (p < 0.0001) and IVVA (P<0.0001) were significantly associated with the log of the F4-specific IgA immune response (R-squared = 41%). i.e. 41% of the variability in the F4-specific log of IgA-ASC is explained by the dose and IVVA categories.

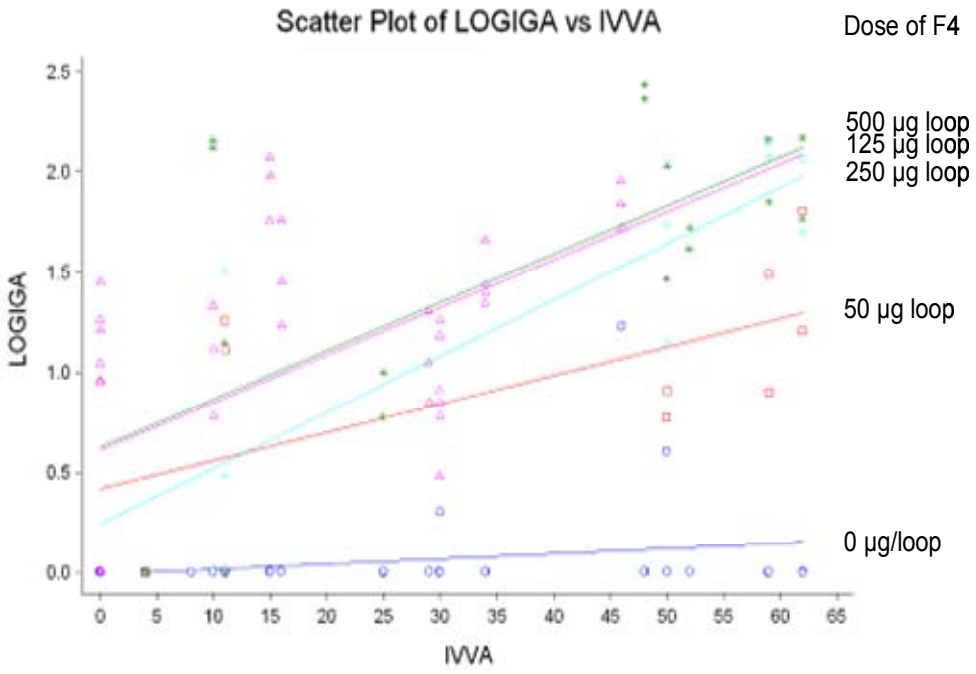
UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOGIGA

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.11962	0.09950	1.20	0.2318	
DOSE	0.00236	3.445E-04	6.86	0.0000	1.0
IVVA	0.01243	0.00270	4.61	0.0000	1.0

R-SQUARED	0.4191	RESID. MEAN SQUARE (MSE)	0.37507
ADJUSTED R-SQUARED	0.4086	STANDARD DEVIATION	0.61243

SOURCE	DF	SS	MS	F	P
REGRESSION	2	30.0373	15.0187	40.04	0.0000
RESIDUAL	111	41.6327	0.37507		
TOTAL	113	71.6700			

CASES INCLUDED 114 MISSING CASES 0


Scatter Plot of LOGIGA vs IVVA
LOGIGA
IVVA
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Dose of F4
500 µg loop
125 µg loop
250 µg loop
50 µg loop
0 µg/loop

Analysis 2. All pigs were examined except those which had previously been exposed to ETEC F4, and thus had immune responses in their control loops. These pigs were therefore categorized as secondary (2°) responders. The 2° responders (cohorts 429, 430, 491) were removed from the analysis to see the effect of dose and IVVA on the log of the F4-specific IgA immune response.

Result – The removal of 2° responders shows that both dose (p < 0.0001) and IVVA (P<0.0001) were significantly associated with the log of the F4-specific IgA-ASC immune response. R-squared = 42%, thus the change was minimal relative to analysis 1.

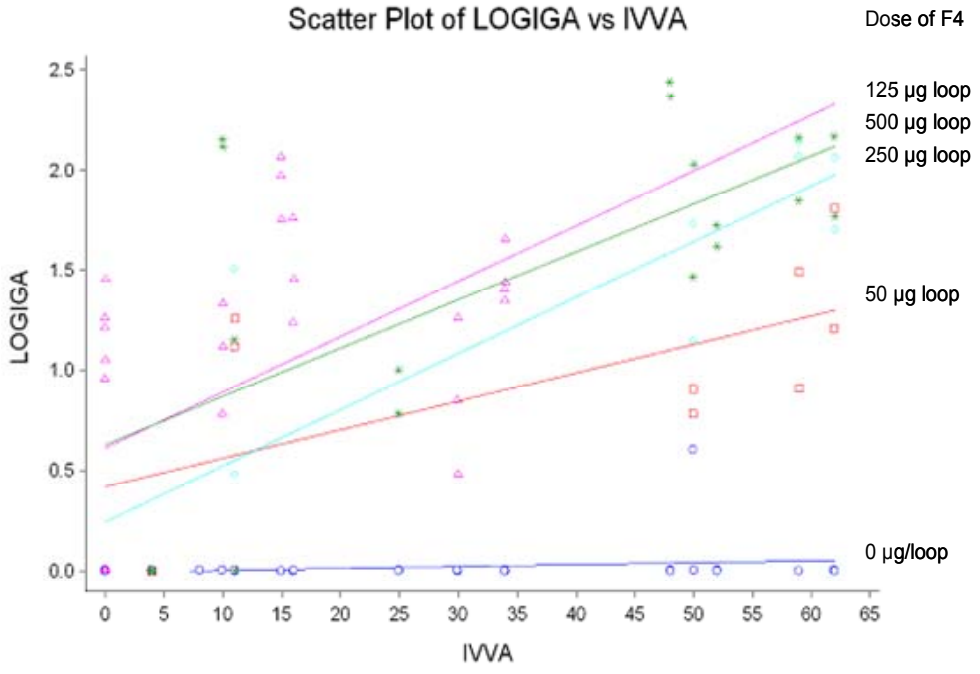
UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgA

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.11431	0.10313	1.11	0.2704	
DOSE	0.00240	3.570E-04	6.72	0.0000	1.0
IVVA	0.01114	0.00280	3.98	0.0001	1.0

R-SQUARED	0.4267	RESID. MEAN SQUARE (MSE)	0.38728
ADJUSTED R-SQUARED	0.4150	STANDARD DEVIATION	0.62232

SOURCE	DF	SS	MS	F	P
REGRESSION	2	28.2515	14.1257	36.47	0.0000
RESIDUAL	98	37.9539	0.38728		
TOTAL	100	66.2054			

CASES INCLUDED 101 MISSING CASES 0



Analysis 3. All loops from 21 pigs were examined for the effect of dose and IVVA on the F4-specific immune response measured by taking the log of the F4-specific IgG ASC.

Result – Both dose (p < 0.0001) and IVVA (P < 0.0001) are significantly associated with the log of the F4-specific IgG ASCs. R-squared = 32%.

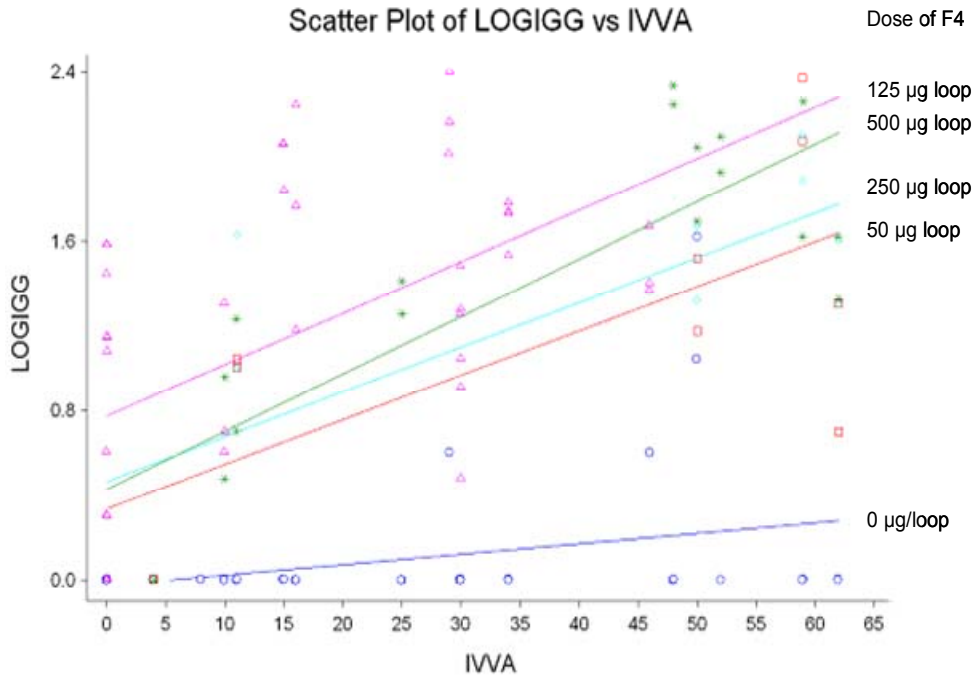
UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgG

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.23728	0.10922	2.17	0.0319	
DOSE	0.00199	3.781E-04	5.26	0.0000	1.0
IVVA	0.01289	0.00296	4.36	0.0000	1.0

R-SQUARED	0.3319	RESID. MEAN SQUARE (MSE)	0.45193
ADJUSTED R-SQUARED	0.3199	STANDARD DEVIATION	0.67226

SOURCE	DF	SS	MS	F	P
REGRESSION	2	24.9197	12.4598	27.57	0.0000
RESIDUAL	111	50.1642	0.45193		
TOTAL	113	75.0839			

CASES INCLUDED 114 MISSING CASES 0



Analysis 4. The 2° responders were removed from the analysis to see the effect of dose and IVVA on the log of the F4-specific IgG immune response.

Result – Removal of secondary responders does not result in a substantial change of the results reported in analysis 3. Both dose (p < 0.0001) and IVVA (P < 0.0001) are significantly associated with the log of the F4-specific IgG ASCs. R-squared = 34%.

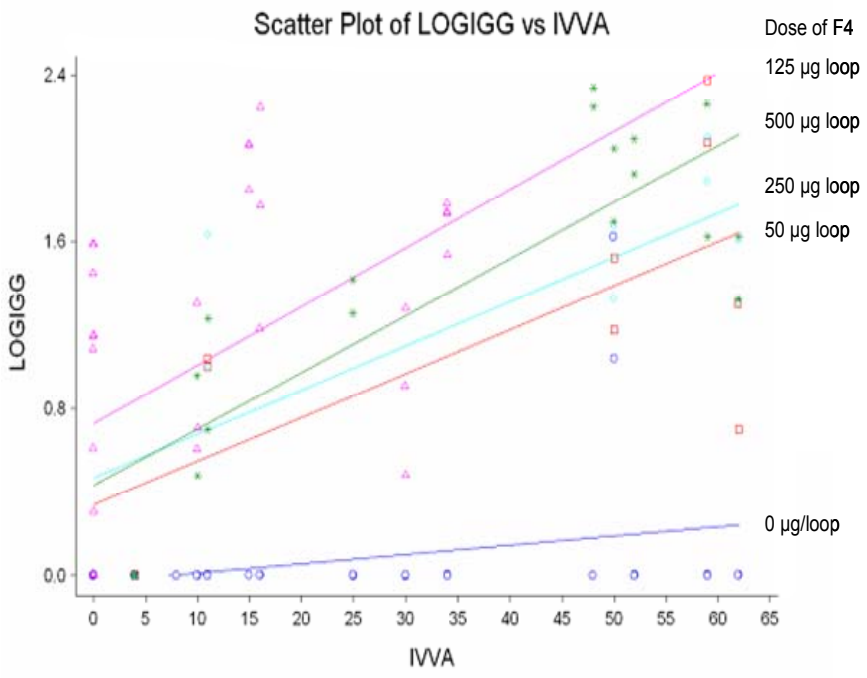
UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgG

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.20517	0.10930	1.88	0.0635	
DOSE	0.00199	3.783E-04	5.27	0.0000	1.0
IVVA	0.01223	0.00296	4.13	0.0001	1.0

R-SQUARED	0.3567	RESID. MEAN SQUARE (MSE)	0.43497
ADJUSTED R-SQUARED	0.3436	STANDARD DEVIATION	0.65953

SOURCE	DF	SS	MS	F	P
REGRESSION	2	23.6398	11.8199	27.17	0.0000
RESIDUAL	98	42.6275	0.43497		
TOTAL	100	66.2673			

CASES INCLUDED 101 MISSING CASES 0



Analysis 5. For all cohorts the non-immunized loops were removed from the data set to permit the examination of the effect of dose and IVVA on the log of the F4-specific IgA in immunized loops only. Interestingly increased doses of F4 Ag was not always associated with an upward shift in the dose response curve (i.e. the order from lowest to highest curve was 0, 50, 250, 500, and 125). Therefore, these findings suggest that the effect of dose was inconsequential to the strength of the F4-specific immune response. Rather it was important if the loop was immunized with the F4 Ag as apposed to not being vaccinated with the protein.

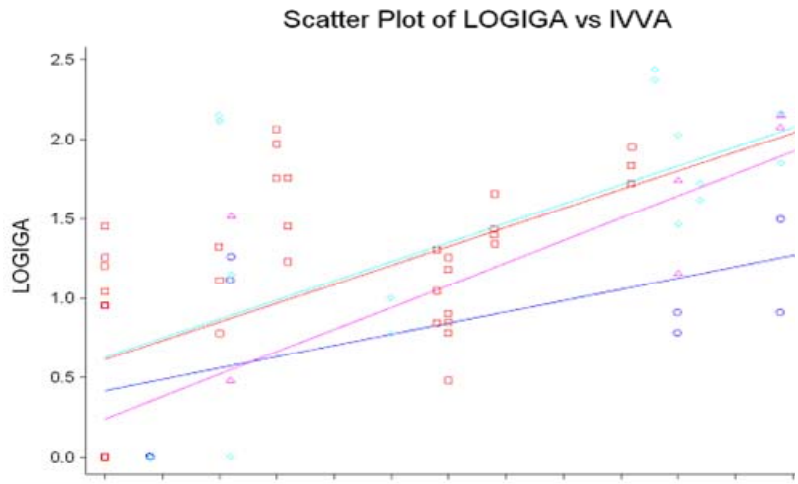
Result – Using only the data from loops administered various doses of F4 AG, only the IVVA (P < 0.0001) was significantly associated with the log of the IgA-ASC immune response. R-squared = 41%. Dose however was not associated with the log of the IgA-ASC immune response (P = 0.13). Removal of the non-immunized loops did not change the R-squared value.

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.49127	0.12001	4.09	0.0001	
DOSE 1.1	6.124E-04	4.015E-04	1.53	0.1314	
IVVA 1.1	0.01977	0.00296	6.67	0.0000	

R-SQUARED	0.4238	RESID. MEAN SQUARE (MSE)	0.31366
ADJUSTED R-SQUARED	0.4084	STANDARD DEVIATION	0.56005

SOURCE	DF	SS	MS	F	P
REGRESSION	2	17.2991	8.64956	27.58	0.0000
RESIDUAL	75	23.5244	0.31366		
TOTAL	77	40.8235			

CASES INCLUDED 78 MISSING CASES 0



Analysis 6. For all cohorts the non-immunized loops were removed from the data set to examine the effect of dose and IVVA on the log of the F4-specific IgG immune response.

Result – Using only the data from loops administered various doses of F4 AG, only the IVVA (P < 0.0001) was significantly associated with the log of the IgG-ASC immune response. R-squared = 36%. Dose however was not associated with the log of the IgG-ASC immune response (P = 0.87). Removal of the non-immunized loops did change the R-squared value.

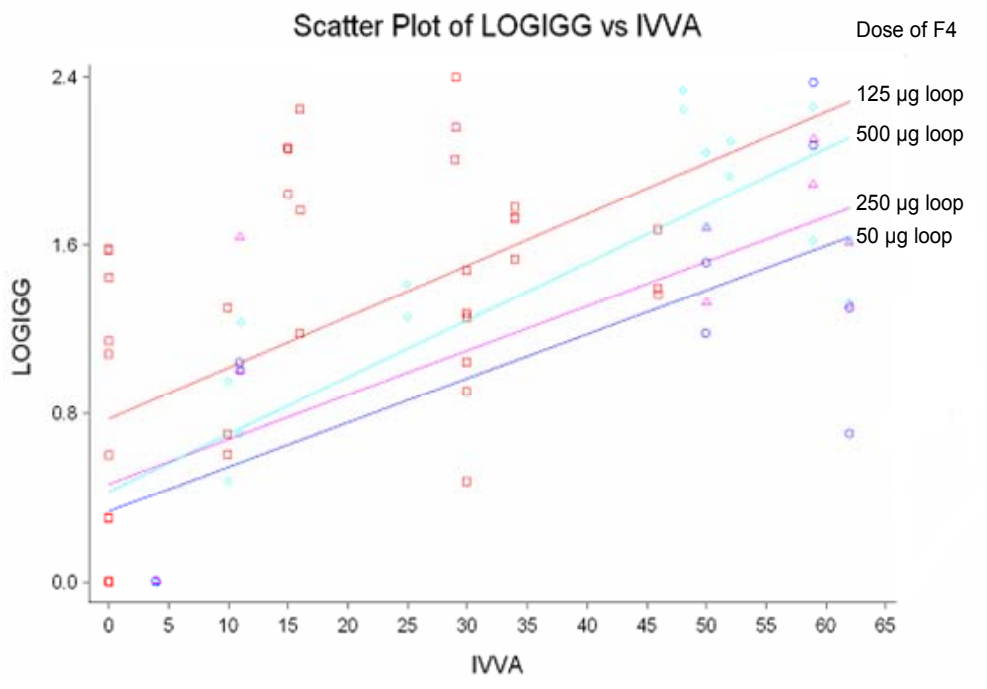
UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgG

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.71710	0.12326	5.82	0.0000	
DOSE 1.1	-6.575E-05	4.124E-04	-0.16	0.8738	
IVVA 1.1	0.02009	0.00304	6.60	0.0000	

R-SQUARED	0.3792	RESID. MEAN SQUARE (MSE)	0.33087
ADJUSTED R-SQUARED	0.3627	STANDARD DEVIATION	0.57522

SOURCE	DF	SS	MS	F	P
REGRESSION	2	15.1611	7.58055	22.91	0.0000
RESIDUAL	75	24.8155	0.33087		
TOTAL	77	39.9766			

CASES INCLUDED 78 MISSING CASES 0



Analysis 7. All cohorts were examined regarding the effect of dose on the F4-specific IgA-ASC immune response, using Kruskal-Wallis nonparametric one-way ANOVA.

Result – Only the non-immunized (dose of 0 ug/ml F4 Ag) loops were noticeably different in the strength of their F4-specific IgA-ASC immune response relative to immunized loops (50, 125, 250, and 500 ug/ml F4 Ag). Amongst immunized loops, there was no significant difference in the F4-specific IgA-ASC immune response.

KRUSKAL-WALLIS ONE-WAY NONPARAMETRIC AOV FOR IgA BY DOSE

DOSE	MEAN RANK	SAMPLE SIZE
0	27.9	36
50	63.5	10
125	66.0	40
250	76.9	10
500	83.8	18
TOTAL	57.5	114

KRUSKAL-WALLIS STATISTIC 50.7451
P-VALUE, USING CHI-SQUARED APPROXIMATION 0.0000

PARAMETRIC AOV APPLIED to RANKS

SOURCE	DF	SS	MS	F	P
BETWEEN	4	51032.1	12758.0	22.21	0.0000
WITHIN	109	62606.9	574.375		
TOTAL	113	113639			

TOTAL NUMBER OF VALUES THAT WERE TIED 77
MAX. DIFF. ALLOWED BETWEEN TIES 0.00001

CASES INCLUDED 114 MISSING CASES 0

COMPARISONS OF MEAN RANKS OF IGA BY DOSE

DOSE	MEAN RANK	HOMOGENEOUS GROUPS
500	83.778	I
250	76.900	I
125	66.000	I
50	63.450	I
0	27.875	.. I

THERE ARE 2 GROUPS IN WHICH THE MEANS ARE
NOT SIGNIFICANTLY DIFFERENT FROM ONE ANOTHER.

REJECTION LEVEL 0.050
CRITICAL Z VALUE 2.81
CRITICAL VALUES OF DIFFERENCES VARY BETWEEN
COMPARISONS BECAUSE OF UNEQUAL SAMPLE SIZES.

Analysis 8. All cohorts were examined regarding the effect of dose on the F4-specific IgG-ASC immune response, using Kruskal-Wallis nonparametric one-way ANOVA.

Result – Only the non-immunized (dose of 0 ug/ml F4 Ag) loops were noticeably different in the strength of their F4-specific IgG-ASC immune response relative to immunized loops (50, 125, 250, and 500 ug/ml F4 Ag). Amongst immunized loops, there was no significant difference in the F4-specific IgG-ASC immune response.

KRUSKAL-WALLIS ONE-WAY NONPARAMETRIC AOV FOR IgG BY DOSE

DOSE	MEAN RANK	SAMPLE SIZE
0	26.9	36
50	65.8	10
125	69.9	40
250	72.5	10
500	78.1	18
TOTAL	57.5	114

KRUSKAL-WALLIS STATISTIC 48.8963
P-VALUE, USING CHI-SQUARED APPROXIMATION 0.0000

PARAMETRIC AOV APPLIED to RANKS

SOURCE	DF	SS	MS	F	P
BETWEEN	4	50341.6	12585.4	20.79	0.0000
WITHIN	109	65998.4	605.490		
TOTAL	113	116340			

TOTAL NUMBER OF VALUES THAT WERE TIED 78
MAX. DIFF. ALLOWED BETWEEN TIES 0.00001

CASES INCLUDED 114 MISSING CASES 0

COMPARISONS OF MEAN RANKS OF IGG BY DOSE

DOSE	MEAN RANK	HOMOGENEOUS GROUPS
500	78.083	I
250	72.550	I
125	69.900	I
50	65.800	I
0	26.944	.. I

THERE ARE 2 GROUPS IN WHICH THE MEANS ARE
NOT SIGNIFICANTLY DIFFERENT FROM ONE ANOTHER.

REJECTION LEVEL 0.050
CRITICAL Z VALUE 2.81
CRITICAL VALUES OF DIFFERENCES VARY BETWEEN
COMPARISONS BECAUSE OF UNEQUAL SAMPLE SIZES.

Analysis 9. Descriptive statistics for the F4-specific IgA-ASC seen in loops as a function of dose. All pigs were included.

Result – As the dose was increased from 0 to 500 ug/ml of F4 Ag, the F4-specific IgA- ASC response also increased. However based on analysis 7, non-immunized loops demonstrated significantly lower responses than did the immunized loops. However, the differences among the immunized loops were not statistically significant.

DESCRIPTIVE STATISTICS FOR DOSE = 0

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGA	0.0000	0.0000	0.0000

DESCRIPTIVE STATISTICS FOR DOSE = 50

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGA	4.5000	10.500	21.250

DESCRIPTIVE STATISTICS FOR DOSE = 125

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGA	3.7500	14.000	27.750

DESCRIPTIVE STATISTICS FOR DOSE = 250

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGA	2.2500	41.000	115.75

DESCRIPTIVE STATISTICS FOR DOSE = 500

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGA	9.0000	55.500	142.00

Analysis 10. Descriptive statistics for the F4-specific IgG-ASC seen in loops as a function of dose. All pigs were included.

Result – As the dose was increased from 0 to 500 ug/ml of F4 Ag, the F4-specific IgG- ASC response also increased. However, only non-immunized loops were significantly lower than all other immunized loops. Among immunized loops no statistically significant difference in the F4-specific IgG-ASC immune response occurred.

DESCRIPTIVE STATISTICS FOR DOSE = 0

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGG	0.0000	0.0000	0.0000

DESCRIPTIVE STATISTICS FOR DOSE = 50

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGG	3.7500	13.000	54.250

DESCRIPTIVE STATISTICS FOR DOSE = 125

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGG	4.0000	19.500	53.750

DESCRIPTIVE STATISTICS FOR DOSE = 250

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGG	7.5000	30.500	54.500

DESCRIPTIVE STATISTICS FOR DOSE = 500

VARIABLE	1ST QUARTI	MEDIAN	3RD QUARTI
IGG	8.0000	34.000	113.25

Analysis 11. All pigs except 2° responders were examined for the effects of dose, IVVA and maternal antibody on the F4-specific log of the IgA-ASC response.

Result - After accounting for the effects of both the IVVA and dose, there was a significant effect of the maternal antibody concentration upon the log of the F4-specific IgA-ASC seen in the loops (P = 0.004). For each log increase in maternal antibody, the IgA-ASC content of the loops decreased by 0.46 logs (see coefficient for logMA – the log of the maternal antibody concentration)

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgA

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.93317	0.29662	3.15	0.0022	
DOSE	0.00252	3.465E-04	7.28	0.0000	1.0
IVVA	0.01080	0.00270	4.00	0.0001	1.0
LOGMA	-0.45805	0.15634	-2.93	0.0042	1.0

R-SQUARED	0.4733	RESID. MEAN SQUARE (MSE)	0.35947
ADJUSTED R-SQUARED	0.4570	STANDARD DEVIATION	0.59956

SOURCE	DF	SS	MS	F	P
REGRESSION	3	31.3371	10.4457	29.06	0.0000
RESIDUAL	97	34.8682	0.35947		
TOTAL	100	66.2054			

CASES INCLUDED 101 MISSING CASES 0

Analysis 12. All pigs except 2° responders were examined for the effects of dose, IVVA and maternal antibody on the F4-specific log of the IgG-ASC response.

Result - After accounting for the effects of both the IVVA and dose, there was a trend for the maternal antibody concentration to affect the log of the F4-specific IgG-ASC seen in the loops (P = 0.09). For each log increase in maternal antibody, the IgG-ASC content of the loops decreased by 0.30 logs (see coefficient for logMA – the log of the maternal antibody concentration)

UNWEIGHTED LEAST SQUARES LINEAR REGRESSION OF LOG IgG

PREDICTOR VARIABLES	COEFFICIENT	STD ERROR	STUDENT'S T	P	VIF
CONSTANT	0.73464	0.32297	2.27	0.0251	
DOSE	0.00207	3.773E-04	5.50	0.0000	1.0
IVVA	0.01201	0.00294	4.09	0.0001	1.0
LOGMA	-0.29617	0.17022	-1.74	0.0851	1.0

R-SQUARED	0.3762	RESID. MEAN SQUARE (MSE)	0.42616
ADJUSTED R-SQUARED	0.3569	STANDARD DEVIATION	0.65281

SOURCE	DF	SS	MS	F	P
REGRESSION	3	24.9299	8.30996	19.50	0.0000
RESIDUAL	97	41.3375	0.42616		
TOTAL	100	66.2673			

CASES INCLUDED 101 MISSING CASES 0