Application of Polymerase Chain Reaction (PCR) and Enzyme Linked Immunoassays for the Identification of Enterotoxigenic *Escherichia coli* Bacteria in Piglet Feces and Antibodies in Pig Serum.

A Thesis

Submitted to the Faculty

Of

Graduate Studies
The University of Manitoba

By

Steven Christopher Cole

In Partial Fulfillment of the
Requirements of the Degree

Of

Masters of Science

Department of Animal Science

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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APPLICATION OF POLYMERASE CHAIN REACTION (PCR) AND ENZYME LINKED IMMUNOASSAYS FOR THE IDENTIFICATION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* BACTERIA IN PIGLET FECES AND ANTIBODIES IN PIG SERUM

BY

STEVEN CHRISTOPHER COLE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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Abstract

Steven Christopher Cole. MSc., The University of Manitoba, September 2001. The Application of Polymerase Chain Reaction (PCR) and Enzyme Linked Immunoassays for the Identification of Enterotoxigenic *Escherichia coli* Bacteria in Piglet Feces and Antibodies in Pig Serum. Major Professor: R.R. Marquardt.

Enterotoxigenic strains of *E. coli* are often causative agents for watery diarrhea in pigs, most notably in neonatal and early-weaned pigs. The result of this type of infection is often a decrease in weight gain and in serious cases, it can lead to mortality of weaker pigs. In recent years there has been public pressure in the livestock industry to ban the use of antibiotics for subtherapeutic and prophylactic applications. Such a move in the industry would lead to higher morbidity and mortality rates at the cost of trying to prevent the emergence of antibiotic resistant strains of bacteria. In order to confront this problem researchers world wide have been looking for alternative therapies that circumvent the antibiotic prohibition laws that are undoubtedly coming to North America while still providing protection for livestock. One alternative to the use of antibiotics is the provision of therapeutic antibodies in pig feed to prevent and treat diarrheal disease caused by specific strains of enterotoxigenic *E. coli.* The mode of action of these antibodies is the prevention of colonization of the bacteria by binding to their adhesive fimbria, thereby allowing the normal passage of waste through the gut to flush out the pathogenic strains of *E. coli.* The mode of action for these antibodies is specific and dependant on the structure of the colonizing fimbria. One type of antibody will only be effective against one type of fimbria.

So in order to apply these products effectively they must be applied accurately. Hence there is a need for the accurate and specific diagnosis of the strains that are causing diarrhea.

In order to test and construct a short history of disease exposure for individual pigs, blood serum samples can be tested for the presence and titer of anti-ETEC antibodies with ELIFA. The presence of antibodies to a specific strain of ETEC would indicate exposure to that ETEC, which elicited and immune response. Genetic screening of fecal swabs from scouring pigs is accomplished with the Polymerase Chain Reaction for the genes that express the fimbrial adhesion particles on the cell surface of the bacteria. This thesis is concerned with the genetic identification of five strains of enterotoxigenic *E. coli* (ETEC) by their fimbrial genes, namely K88ac (F4), K99 (F5), 987p(F6), F41 and F18 (F107). The development of an antigen enzyme-linked immunofluorescent assay (ELIFA) is examined along with the application of an ELISA for the identification of anti-ETEC antibodies in pig serum. The application of all three techniques would constitute a total detection system for the identification of both fimbrial genes and their fimbria expressed *in vitro*.

The ELISA assay for the presence of anti-ETEC antibodies in pig serum samples was effective in testing for the presence of complementary antibodies but was never applied beyond preliminary trials. The PCR assays too were successful in testing for the presence of ETEC in the fecal samples of pigs specifically inoculated with control strains of bacteria. The ELIFA for the presence of target antigens was also successful but proved very difficult to optimize reproduce and apply on a large scale. The PCR assay is here proven to be a very useful tool to test for the presence of target bacterial strains with excellent reproducibility, versatility and sensitivity. The ELISA is also a valid and useful means of identifying anti-

ETEC antibodies in serum samples but the ELIFA used to test for the presence of target antigens is unsuitable for large-scale testing of bacterial samples.

Acknowledgements

I would like to thank my committee members, Dr. R.R. Marquardt, Dr. J. House and Dr. P. Loewen for all their vital assistance supplying me with the tools and advice I needed. Dr. A. Owusu-Asiedu, thanks for all the pigs, and Lin Fang, thanks for showing me the secrets of ELISA methodology. Thanks to Dr. Fairbrother and his staff at the University of Montreal department of Veterinary Medicine (GREMIP) for the fundamentals of the PCR. And of course I would like to thank all of my colleagues in the department especially Mark Zhang, on whom I have relied for advice, insight and support. This research was funded by a grant from the Natural Sciences and Engineering Research Council of Canada.

Dedication

This work is dedicated to all those who helped me keep it together when all this was driving me mad, to my Lady Dar, my family, Mum, Jim, Aunt Rose, Uncle Ray, Thelma and Morgan. And to my friends who have been like family, Aaron, I'm always feelin' pubish. And finally to Terry Pratchett , Sam Vimes, and all the beings of the AMCW.

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Table of Abbreviations

Abbreviation	Definition
ADP	Adenine diphosphate
bp	Base pairs
CDP	Cytosine diphosphate
CFU	Colony forming units
CSCA	Chloroform separated egg-yolk antibodies
dATP	Deoxyadenine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
DIFT	Direct epifluorescence filtration technique
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
dTTP	Deoxytyrosine triphosphate
dTTP	Deoxythymine diphosphate
ELIFA	Enzyme linked immunofluorescent assay
ELISA	Enzyme linked immunosorbent assay
em	Emission wavelength
ETEC	Enterotoxigenic Escherichia coli
ex	Excitation wavelength
FDP	Flourescein diphosphate
GDP	Guanine diphosphate
HRP	Horse radish peroxidase enzyme
LT	Heat labile enterotoxin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ssDNA	Single stranded DNA
STa	Heat stable enterotoxin a
STb	Heat stable enterotoxin b
Taq Pol	Thermus aquaticus DNA polymerase enzyme
TDP	Thymine diphosphate
Tm	Melting temperature (as applied to primers)

General Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infections detrimental to the performance of swine that can lead to lack of weight gain, weight loss and even death due to complications of dehydration. The bacteria are mainly transmitted by the fecal-oral route to piglets that root in fecal material found in the pens with the primary source being other carrier animals. The bacteria can survive the low pH of the stomach and can then be deposited in the small intestine. There the fimbriae, which are rod-like adhesion particles on the bacterial cell surface, will mediate attachment of the bacteria to the wall of the intestine; this is the prerequisite of colonization and thus infection. ETEC that colonize the gut also produce and secrete enterotoxins whose concentration in the gut increases in proportion to the population of the pathogens. Large quantities of toxins in the gut will effect ion transport in the gut epithelial cells resulting in an electrolyte imbalance in the gut(Gyles, 1994). The net flow of ions is into the gut lumen creating a hypertonic environment that leads to the secretion of water from the epithelial cells into the lumen causing acute cases of watery diarrhea, dehydration and loss of appetite in pigs. There are many forms of toxins produced but they are all classified as being either heat stable or heat labile enterotoxins. Both classes of enterotoxins contain two subunits (A and B) (Spangler, 1992). The B subunit is responsible for binding of the toxin to the target epithelial cells of the small intestine. The A subunit of the heat labile enterotoxin causes ADP ribosylation of adenyl cyclase. This results in the overproduction of cyclic AMP and a net excretion of chloride ions (Guerrant *et al.*, 1974). This electrolyte imbalance causes decreased absorption of sodium ions and so the concentration of sodium chloride in the intestinal lumen will rise leading to a loss of water from the intestinal epithelial cells. The loss of water into the lumen

is the cause of the watery diarrhea and dehydration. The result is the same with the heat stable enterotoxin except that the toxin causes ADP ribosylation of guanylate cyclase that leads to the overproduction of cyclic GMP (Field *et al.*, 1978; Hughes *et al.*, 1978). This causes a reduction in the absorption of the sodium ion from the intestinal lumen resulting in a build up of electrolytes in the lumen and thus a net water loss by the epithelial cells. Watery diarrhea is again a result of a loss of water from the epithelial cells to the lumen.

The use of therapeutic antibodies is highly effective in preventing and treating ETEC induced diarrheal infections (Owusu-Asiedu et al. 2002); they stop the colonization of new bacteria through competitive inhibition of the colonization fimbria. The antibodies irreversibly bind to the fimbrial adhesion particles of the bacteria neutralizing their binding abilities. This will prevent the bacteria from colonizing and reproducing in the gut in sufficient numbers to cause diarrhea. The antibodies themselves can be raised in layer hens through specific vaccination using the fimbrial adhesion particles found on ETEC. Their immune response will lead to the production of immunoglobulin G that can be recovered in the yolks of eggs from these hens. The antibodies, when given orally to pigs up to six weeks of age, can pass though the immature gut and maintain activity against ETEC adhesive fimbria. This prevents colonization allowing the gut to flush the bacteria out with the normal passage of fecal waste. However, the application of the antibodies needs to be accurate as only K88 bacteria will react with K88 antibodies. Therefore, there is a need for accurate diagnosis of the strain of ETEC colonizing the gut.

The polymerase chain reaction is a genetic amplification technique that replicates target segments of DNA many many times. Once complete the PCR products can be

separated by electrophoresis in agarose gel and identified according to its typical molecular weight. The accuracy attributed to the test involves the specificity of the primers for unique DNA sequences within the genes of interest. This means that the primers designed for the diagnosis of K99 fimbrial genes will exclusively prime the polymerase chain reaction for K99 bearing *E. coli*. For the purposes of this thesis there are five sets of initiation and termination primers employed to identify the presence of the fimbrial genes of K88ac(F4), K99 (F5), 987p(F6), F41 and F18 (F107) strains of ETEC.

A short history of disease exposure as pertains to the five ETEC's can be obtained by assaying for the titer of anti-ETEC antibodies in the serum of pigs. This can be achieved using an Enzyme Linked Immunosorbent Assay (ELISA) where fimbrial isolates are bound to a ninety-six well assay plate and allowed to react with antibodies present in the serum. The activity of these antibodies is measured in relation to an appropriately selected reference standard such as that present in commercially available spray dried porcine plasma. In this way any recent infections by ETEC could be identified and used to predict disease cycles that may be seen in progeny animals of a given facility. The specificity of this assay relies on the accurate isolation of the fimbria from a given and pure strain of ETEC as these fimbria are used to coat the assay plates. The same fimbriae are administered in a subunit vaccine to generate the antibodies harvested from the chicken egg yolks and in the assays themselves.

The objective of this thesis is to apply the existing ELISA and PCR technologies to develop a fast functional ETEC diagnostic and verification system. This system can then to be applied to testing pig feces and serum in vivo for the accurate application of therapeutic antibodies to treat ETEC's.

Literature Review

1.1 Introduction:

The focus of this research was to develop assays that can detect specific strains of enterotoxigenic *E. coli* (ETEC) using cellular immune-based or PCR gene based assays. Therefore, the development strategy of the assays to be used in the study must be placed into context relative to their use in the laboratory and in the barn. Two topic areas must be explored to do so. The first is how ETEC cause disease with its collection of virulence factors especially the fimbrial adhesins and the enterotoxins. The second relevant topic area concerns the current methods for detecting and identifying specific strains of *E. coli* and how these relate to the methods that will be employed.

1.2 ETEC in Piglet Diarrhea:

According to Gyles (Gyles, 1994) "...the highest incidence of life-threatening diarrhea occurs during the first 3-5 days of age with less serious peaks occurring later. In diarrhea of the newborn piglet ETEC are frequently the primary and sole infectious cause." It has been observed that K88 strains are the most common until four days of age. Other non-K88 strains of ETEC are the prime suspects from 4 to 14 days of age, and then K88 becomes dominant again starting around 21 days of age (after weaning), (Nagy and Fekete, 1999). During severe outbreaks the morbidity and mortality associated with ETEC in neonates can be high indeed and losses in performance due to scouring can be hard to recoup; the affected pigs loose weight and dehydrate rapidly. Not all piglets will be clinically ill so therapy for the clinical cases as well as prophylaxis for the littermates is valuable. The

fimbriae associated with neonatal specifically adhere to the surfaces of enterocytes in the gut (Gaastra and de Graaf, 1982; Moon, 1990). There are a number of different types of Fimbriae to mediate this binding, the most common of those is referred to as the K88 antigen (K88ab and K88ac variants). Officially it is called fimbrial adhesin 4 (F4). F4 is considered to be exclusive to pigs and is commonly associated with neonatal diarrhea (Nagy and Fekete, 1999) accounting for 40 to 60% of cases. F6 (987P) is also specific to ETEC that infect pigs but is less common than K88. F5 (K99) and F41 commonly occur together and are predominantly associated with calves and sheep although they have also been known to infect pigs (Gyles, 1994). Neonatal diarrhea caused by the non-K88 strains has been shown to account for 20 to 30% of diarrhea cases (Nagy and Fekete, 1999). K88 most often produces both LT and ST enterotoxins (Wilson and Francis, 1986; Harel *et al.* 1990). K99, F41 and 987P are associated with STa and/or STb enterotoxins (Fairbrother, 1992).

This section will consider how piglets become infected with these pathogens. The environment of pigs in the uterus is normally sterile. As a result, pigs are born with no active or innate immunity of their own. The immunity they do have is by way of passive antibodies acquired from the colostrum of the dam and their own innate non-specific host defenses. Never the less, the piglet immediately upon birth is exposed to a variety of organisms, the majority of these organisms will be non-pathogenic members of the normal flora associated with the bodies of pigs. These organisms will colonize the skin, respiratory and gastrointestinal tract of the baby pig, thereby improving the nonspecific host defense system. Normally the organisms are shed by the mother in the pen and are found in the feed and airborne dust of the barn. However, the neonate may be exposed to some pathogenic organisms, likewise shed by animals in the barn. This incidentally, can account

for the chronic passage of diseases from one generation to the next within the barn. Further there are no innate defenses to protect the piglet, if the colostrum antibodies of the dam are insufficient to fight off an ETEC diarrheal infection; the reasons for this may be multifactorial. The gastrointestinal tracts of neonatal pigs are of a nearly neutral pH with a low concentration of digestive enzymes, conditions that are favorable for bacterial colonization and persist for a number of hours after birth. Therefore, ETEC bacteria can adhere and multiply in the gut of the pig as easily as the natural flora. The only barrier that protects the piglet against ETEC induced diarrhea is the secretory IgA in the colostrum and any nonspecific host defenses that are working at the time.

There is a condition seen in pigs termed post-weaning diarrhea (PWD), that begins 3-10 days after weaning and was first clearly described by Richards and Fraser (1961). The clinical symptoms of the disease are much the same as for neonatal diarrhea, with loss of weight gain, progressive emaciation, depression, loss of appetite and dehydration. Mortality can reach 25% without proper medication (Richards and Fraser, 1961). There are a number of factors that are thought to promote this condition including early weaning practices, the removal of lacteal protection and exposure to new environments, animals and pathogens (Nagy and Fekete, 1999). This state of affairs is of definite consequence to the hog industry accounting for economic losses due to mortality and reduced growth rates (van Beers-Schreurs *et al*.,1992) as well as being a major cause of postweaning mortality in many production facilities.

The bacteria found in postweaning diarrhea have adhesive fimbria that keep them firmly attached to the intestinal mucosa thus preventing their removal through the normal

waste excretion processes (Svendsen *et al.*, 1977). The best known and most common is the K88 pilus or fimbriae (Jones and Rutter, 1972) which is associated with the main PWD serogroups. Three antigenic variants of K88 (K88ab, K88ac, and K88ad) facilitate the adhesion of ETEC to the villi throughout the small intestine. The other pili associated with PWD are K99 (F5), 987P (F6), and F41 but these are less common (Nakazawa *et al.*, 1987; Nagy *et al.*, 1990). In addition F107 (F18) has also been linked to PWD. The suggestion has been made that K88 bearing *E. coli* are more likely to cause diarrhea about 4 days after weaning whereas the other pilus strains tend to initiate their effect at 7-10 days postweaning (Nagy and Fekete 1999). This is thought to be a result of the changes that occur in the gut after weaning, a factor that may lead to alteration of the intestinal mucosa and associated epithelial receptors.

Most cases of postweaning diarrhea are caused by ETEC so by definition they produce one or a combination of either heat labile (LT), heat stable (Sta) and/or heat stable (STb) enterotoxins (Morris and Sojka, 1985). These toxins do not directly induce cytotoxic effects *per se*; rather they interfere with the regulatory functions for ion exchange, which leads to fluid secretion into the lumen of the small intestine causing watery diarrhea.

1.3 Virulence Factors:

According to Warren and Jawetz (1998) virulence is "a quantitative measure of pathogenicity and is measured by the number of organisms required to cause disease". The pathogenicity or virulence is dependent on the virulence factors which may include adhesive fimbriae to aid in colonization on target tissues, antiphagocytic capsules, invasive properties,

motility, and/or the capacity to produce various classes of toxins. For this thesis the most relevant virulence factors are the adhesive fimbriae and the enterotoxins which are most often associated with diarrhea in the young pig.

1.3.1 Enterotoxins:

The presence of enterotoxins was first established in pigs by the ligated intestine technique. The secretions of *E. coli* suspected of causing diarrhea were exposed to samples of ligated pig intestine and their secretions were able to cause fluid loss from the epithelium of the intestine (Moon *et al.*, 1966; Gyles and Barnum, 1967; Smith and Halls, 1967a). There are two classes that cause these responses, the first is the heat labile (LT) class and the other is the heat stable class (ST). The ST class represents two subclasses, STI and STII (also known as STa and STb). Burgess *et al.* (1978) reported that STa is soluble in methanol and active in the mouse intestine while STb is insoluble in methanol and active in the intestine of weaned pigs. Within the STI subclass there are three variants, STIa with 18 amino acids, STIb with 19 amino acids and EAST1 (Moseley *et al.*, 1983). LT represents two subclasses as well; these are LTI and LTII with LTI being the most common. Any strain of *E. coli* producing one or more of these toxins is classed as an ETEC. LT and ST react with different receptor sites on the target cells but their activities result in the same symptoms and will not cause direct pathological lesions or morphological changes on the mucosa (Nagy and Fekete, 1999).

1.3.2 Heat Labile Enterotoxins LT:

Structurally the LT toxin is an oligomeric protein approximately of 88 kDa, within that there are two subunits. The A subunit carries the active region of the toxin. The B subunit is a stable arrangement of five smaller proteins (Spangler, 1992) held together by hydrogen bonds and salt bridges in a doughnut shaped configuration (Spangler, 1992; Mekelanos *et al.*, 1979). The A subunit in the functional form, "sits" above the hole or aqueous channel of the doughnut conformation for the five B subunits (Sixma *et al.*, 1993). The five B subunits of the toxin will bind to the GM1 ganglioside receptor on the target cell (Sixma *et al.*, 1992; O'Brien *et al.*, 1996., Nagy and Fekete, 1999) leading to a conformational change in the toxin molecule. Once the B subunits are fixed to their receptors on the target cell at the villus tip, the A subunit will translocate through the channel into the cell where they produce their enzymatic activity.

The toxin affect the enzymatic activity of adenosine-diphospho-ribosyl transferase (Fishman, 1990). The enzyme transfers ADP-ribose from nucleotide adenine dinucleotide (NAD) to the arginine residue in the 42-kDa stimulatory guanine nucleotide-binding regulatory protein subunit ($D_{s\alpha}$) of the adenylate cyclase system (Mekelanos *et al.*, 1979). ADP ribosylation results in the inability of the affected cells to turn off the system and persistent elevation of cAMP results in levels several hundred fold above normal in cells exposed to the toxin (Guerrant *et al.*, 1974). Activity requires presence of guanine triphosphate and *in vitro* is augmented by ADP-ribosylation factors (ARF's) (Moss and Vaughan, 1991). To recap, the A subunit is activated through association with ARF-GTP complex and it transfers ADP-ribose from NAD to $G_{s\alpha}$. ADP-ribosylated $G_{s\alpha}$ binds GTP and complexes with the catalytic unit of adenylate cyclase to form an active complex that

converts ATP to cAMP. The complex remains active due to the ADP-ribosylation inhibiting the normal intrinsic GTPase activity of $G_{s\alpha}$. Further, ADP-ribosylation of $G_{s\alpha}$ results in increased affinity of $G_{s\alpha}$ for GTP and decreased affinity for GDP, which promotes maintenance of the active state of the adenylate cyclase complex (Gyles, 1994). ADP-ribosylation of $G_{s\alpha}$ occurs in the brush border membrane of the intestinal epithelial cells. Physiologically speaking the effect of LT is watery diarrhea with elevated levels of cAMP being the cause. Normal intestinal ion transport mechanisms are maintained by phosphorylation of proteins through activation of protein kinases. Excessive stimulation of protein kinases is suspected to lead to electrolyte disturbances that characterize LT mediated diarrhea (Gyles, 1994). LT leads to increased excretion of Cl^- ions from crypt cells and impaired Na^+ and Cl^- absorption at the tips of the villi (Moriarty and Turnberg, 1986). Water follows the electrolytes into the lumen and watery diarrhea results. There is no detectable histological damage as LT merely over stimulates the normal regulatory mechanisms of the affected cells.

Holmes and Twiddy (1983) as well as Lindholm (Lindholm *et al.*, 1983) suggest that immunization with intact LT leads to antibodies that react strongly with the B subunit and weakly or not at all with the A subunit.

1.3.3 Heat Stable Enterotoxin STI (STa):

STI has a molecular weight of approximately 2000 (Lasure *et al.*, 1983), is heat stable up to 100°C for up to 15 minutes and soluble in water and organic solvents. It will resist proteolytic enzymes (Smith and Halls, 1967b). It is acid resistant but susceptible to alkaline

pH and is deactivated by reducing/oxidizing agents that disrupt disulfide bonds (Robertson *et al.*, 1983).

STI binds to the receptors of the brush borders of intestinal epithelial cells. Binding of toxin is maximal in villus preparations and decreases from the villus to the crypt. Binding is regulated by nucleotides that also regulate guanylate cyclase activity in the pig and rat intestinal brush border membranes (Gazzano *et al.*, 1991).

The function of STI is to activate particulate guanylate cyclase activity in the brush border of intestinal epithelial cells in the jejunum and ileum. It leads to elevated cGMP levels based on specific receptors (Field *et al.*, 1978; Hughes *et al.*, 1978). Young pigs are more sensitive (show higher rates of cGMP stimulation in enterocytes) than older pigs but no differences are seen in guanylate cyclase activity in STI-stimulated intestinal epithelial brush border membrane in seven day to seven-week-old pigs (Robertson, 1988). This would indicate that sensitivity to STI is not a factor that is determined by age susceptibility thus it is not reliant on changes to receptors in the maturing gut tissue. The rate of guanylate cyclase stimulation by STI is rapid with maximal levels of cGMP being seen within five minutes. The result is increased fluid secretion by an as yet unknown mechanism. Absorption of Cl^- and Na^+ into the villus tips is impaired and the secretion of Cl^- is stimulated in the crypt cells, this leads to fluid build up in the lumen of the small intestine (Forte *et al.*, 1992). STI works in both the small and large intestine, it causes fluid loss in the small intestine and the problem is further complicated by reduced capacity for fluid absorption in the large intestine (Mezoff *et al.*, 1992). Receptor activation by STI sets off a cascade of reactions that lead to the release of fluid and electrolytes through the stimulation of particulate guanylyl cyclase

that increases the intracellular cGMP content. The stimulation is likely due to phosphorylation of the STI receptor on guanylate cyclase or a related protein by protein kinase C (Crane *et al.*, 1992). Following the increase in intracellular cGMP there is an increase in intracellular calcium and activation of phosphatidyl inositol pathway (de Jonge *et al.*, 1986). STI is potent, fast acting and its effects are of short duration. It can also cause a change in the myoelectric activity of the small intestine resulting in loss of the normal peristaltic activity (Giannella, 1983).

EAST-1 is a close analog of STI and its activity is virtually the same. Robertson *et al.*, (1983) suggested that antibodies to these can only be produced when the toxin is conjugated to a carrier protein.

1.3.4 Heat Stable Enterotoxin STII (STb):

STII is a 48 amino acid peptide (Fujii *et al.*, 1991). Given its small molecular size it must be conjugated with a carrier to evoke an immune response. There is no cross-reactivity between anti-STb antibodies and the STI or CT toxins (Hitostsubashi *et al.*, 1992a). The mode of action of STb is largely unknown. STII has no effect on the levels of cAMP or cGMP nor does it alter the Na^+ or Cl^- unidirectional fluxes (Weikel *et al.*, 1992a). It has been shown, however, to open G-linked receptor operated calcium channels in the plasma membrane (Dreyfus *et al.*, 1993). It will also cause mild histological damage of the intestinal epithelium that may lead to impaired absorption of fluids (Whipp *et al.*, 1987). STII has been shown to increase motility in the mouse intestine by direct action on ilial muscle cells (Hitotsubashi *et al.*, 1992b).

STII is rarely expressed alone and it is almost exclusive to ETEC that infect pigs. The presence or absence of the gene for STII makes little or no difference on the organisms ability to induce diarrhea in the neonatal pig (Casey *et al.*, 1993). The conclusion is that STII does not contribute significantly to ETEC induced diarrhea in neonatal pigs.

1.3.5 Fimbriae:

One of the prerequisites for an ETEC infection as with most *E. coli* infections is attachment; the bacteria have to have some way of attaching to the host tissues (Nagy and Fekete, 1999). Adhesion is considered a requisite for ETEC infection because without it the bacteria would be flushed out by peristaltic propulsion through the intestinal tract, a mechanism of nonspecific host defense against pathogens. The adhesive molecules of the ETEC previously discussed are termed the fimbriae, surface molecules which are made up of a number of structural and adhesive subunits with specific binding properties (Gyles, 1994). *E. coli* makes contact with the epithelial surfaces of the target organ, the small intestine in the case of ETEC, where they seek out and bind their specific receptors which is a prerequisite for this type of infection. Once bound to the intestinal enterocytes the bacteria can replicate in vast numbers causing clinical *E. coli* induced diarrhea.

This research is exclusively concerned with the diagnostic identification of K88, K99, 987P, F41 and F18 type adhesive fimbria in ETEC strains. K88, K99 and 987P genes are all located on plasmids whereas F41 and F18 genes are located on chromosomes. Morphologically the fimbrial types considered are fibrillar with the exception of 987P and F

18 which are rigid and rod-shaped (Gyles, 1994). The P fimbriae have their binding region on the tip of the fimbrial structure. K88 and K99 express adhesive multivalency meaning the subunits that make up the structure can also bind the specific host receptor (Gyles, 1994).

Attachment of an ETEC strain is mediated by two things, the first is the binding specificity of the fimbria themselves and the presence of their receptor on the target host tissue. Given that idea, it is possible for some strains of pigs to be genetically resistant to ETEC strains like K88 by lacking their receptors or at least by having versions of the receptors with a morphology that the bacteria do not recognize (Rutter *et al.*, 1975; Sellwood *et al.*, 1975). Biochemically the host receptors are glycoproteins and glycolipids (Smyth, 1986; Karlsson *et al.*, 1992; de Graaf and Gaastra, 1994). "The adhesin-receptor interaction occurs in a lectin-like fashion. The receptors are present in mucus, epithelial membranes and basement membranes... The receptor epitope of an oligosaccharide is often an internally located sugar sequence which is exposed such that the fimbrial lectin binds to it in a stereospecific manner " (Gyles, 1994).

The expression of fimbriae can be energetically costly since the production of fimbriae can account for approximately 5-10% of total cellular protein. For this reason it is important to regulate the expression of the fimbrial genes, when they are not needed or will not contribute to the infectiousness. Therefore, they will not be produced when not needed thus saving on protein precursors and energy of production (Gyles, 1994). However ETEC when kept in conditions that approximate the gut in terms of temperature and osmolarity will express fimbria *in vitro* (Smyth and Smith 1992).

1.4 Virulence Factors Associated with Neonatal Diarrhea:

The strains that are normally associated with ETEC in suckling pigs are K88 (K88ab K88ac K88ad) and the so-called non-K88 strains (K99, 987P, and F41) but to a much lesser degree. K88 ETEC, usually K88ac will produce LT in addition to STa or STb enterotoxins (Nagy and Fekete, 1999) whereas the non-K88 strains will produce only STa (Nagy and Fekete, 1999). This will usually occur between 1 and 5 days of age and tends to be severe. The non-K88 strains will cause a milder diarrhea from 4 until 14 days of age. The pathogenesis of the diarrhea, to a large extent, depends on the presence of the receptors for the toxin. Receptors for K88 are glycoproteins and are abundant in newborn pigs and will decrease with age remaining stable throughout weaning and postweaning. K99 receptors decrease with age (Nagy and Fekete 1999) while the number of receptors for 987P increases with age (Nagy and Fekete 1999). F41 receptors are thought to be produced throughout the weaning period (Nagy and Fekete 1999).

1.5 Virulence Factors Associated with Postweaning Diarrhea:

The main adhesive fimbriae seen are, as indicated above, K88ac with K99, 987P, F41 and F18 being isolated to a far lesser degree. These ETEC are known to produce more than one type of adhesin such as K88, F18ac or K88, F41 or even K88, F41 and F18ac (Nagy and Fekete 1999). Those producing LT almost always produce K88 and those that produce STa and/or STb mainly produce F18ac (Nagy and Fekete 1999). K99 bacteria have been found to be associated with LTI and STI (Osek 1999). The same study documented that 987P exhibits the same toxin profile with F18 being mainly associated with LTI or LTI

with STI. It should be noted that PWD ETEC can be found to produce STa, STb, or LT or combinations thereof.

The fimbriae-toxin associations and proportions are not constant, papers may contradict each other in finding different percentages of fimbrial types in ETEC as well as different combinations of toxin associated with these fimbriae (Osek, 1999, Ojeniyi, *et al* 1994 and Nagy and Fekete 1999). The root of the matter is that the profile of virulence factors found in ETEC infections varies widely in presence, prevalence and combination. This is likely based largely on region, what original strains are present, how they acquire their virulence factors and how disease control protocols are applied. Assays for ETEC have been developed that can detect both adhesins and specific toxins. Some of these are discussed in the following section.

1.6 Assay Techniques:

There are a number of assay techniques that can be used to test for the presence of specific and general types of bacteria. There are several types of genetic assays including the polymerase chain reaction and the colony hybridization technique, and among these there are a number of refinements and variations to make them easier, faster and more reliable. There are also immunological assays including immunofiltration, ELISA and agglutination assays that can identify specific types of organisms, all with good result. A whole host of other assays exist to exploit various other aspects of the target organism for identification including membrane filter assays, specific growth media and specific activity assays like the ligated intestine test for enterotoxins. Researchers must decide which of these assays is best for their specific applications.

1.6.1 Genetic Assays:

1.6.1A Polymerase Chain Reaction (PCR):

The PCR is a DNA based amplification technique; practically this means that the reaction is like a DNA photocopier making many copies of a target DNA strand. The resulting DNA copies can be visualized using different methods to confirm the presence or absence of a target organism. The key components required for the PCR are the primers, the DNA polymerase enzyme, the enzyme buffer and the template. Primers start the reaction by providing a short double stranded segment on a single stranded template to initiate and terminate the action of DNA polymerase. For the reaction to be specific, the primers must define a unique sequence within the genome of the target organism(s). The sequences chosen for this research are found in the genes for the fimbrial adhesin particles of K88, K99, 987P, F18 and F41. The target sequences can be cross-referenced through databases like NCBI against other known genome sequences to ensure uniqueness. Once this is established the gene sequences can be processed through a primer design program like Primer 3 which is found on the web site www.-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi . This program can be used to design two oligonucleotide primer sequences, one priming in one direction and the second in he opposite direction. Once the primer sequences are found that will prime the reaction they are once again crosschecked in NCBI to ensure that they are unique to the target. Molecularly, the primers are approximately twenty base pairs long and are complementary for a specific region of the template sequence. Upon hybridization to the target sequence it provides a suitable

initiation complex for DNA polymerase to begin adding complementary bases in a pair-wise fashion to the single stranded primed DNA template. In a given PCR reaction two primers are used to border the gene fragment of interest and initiate the DNA polymerase activity on opposite single stranded DNA templates derived from denaturation of the original double stranded template.

The next vital ingredient is Taq polymerase, the DNA polymerase used in most PCR methods. The enzyme itself is taken from the bacterial strain, *Thermus aquaticus*, whose natural habitat is in hot springs at temperatures of 100°C or more. The enzymes produced by these bacteria are therefore very heat stable, a vital property when considering the heating conditions required for the PCR. The optimal operating temperature for this enzyme is 72°C and its function is to add complementary bases to a single stranded DNA template to yield double stranded DNA. The Taq buffer is also an important component of the assay.

Another component needed for the reaction to proceed is the target strand or template. This is required for the successful reaction but it is on that basis, success or failure of the reaction, that the presence of the target organism is established.

There are also thermal requirements that must be met for the reaction to work. The cycle consists of a denaturation step where double stranded template DNA is broken into single strands, and an annealing step where primers attach to the single stranded DNA template and an elongation step where Taq polymerase (Taq pol) will add the complementary bases to the primed single stranded target. The annealing temperature is

decided by the structural and chemical composition of the primers. The extension step is at 72°C, the optimal temperature for the action of Taq pol.

There are a number of ways to analyze the products of a polymerase chain reaction. In this research the replicated DNA is separated by electrophoresis in agarose gel-stained with a fluorescent dye, When the current is applied and the DNA is drawn through the gel, it picks up the ethidium bromide stain while migrating. The migrating DNA products of the PCR will have more stain than the surrounding gel and thus glow more brightly when exposed to ultraviolet light. This provides the positive or negative result for the PCR.

Another method for detection of PCR products is the use of a Taq Man assay (Oberest *et al.*, 1998). This is an assay technique where an oligonucleotide probe is incorporated into the reaction mixture that has been labeled with two fluorescent dyes, one termed the quencher and the other called the reporter. The "assay exploits the 5'→3' exonuclease activity of *Taq* DNA polymerase to hydrolyze an internal TaqMan probe labeled with a fluorescent reporter dye and a quencher dye. For the intact probe, the quencher dye suppresses the fluorescent emission of the reporter dye due to its spatial proximity on the probe. During PCR, the probe anneals to the target amplicon and is hydrolyzed during extension by the *Taq* DNA polymerase. The hydrolysis reduces the quenching effect and allows for an increase in emission of the reporter fluorescence. This increase is a direct consequence of a successful PCR, whereas the emission of the quencher dye remains constant irrespective of amplification." (Oberest *et al.*, 1998). Once the PCR has been run the results can be visualized and quantified with a fluorescent microplate reader tuned to

excite the specific wavelength of the reporter dye. The intensity of the fluorescency is proportional to the amount of PCR product present.

Another similar yet slightly different technique incorporates a molecular beacon (Robinson and Mueller ; McGown and Su, 1999). This too employs an oligonucleotide probe specific for a region within the target sequence but the difference is in the structure of that oligonucleotide. In this case, it is synthesized with two additional tails on each end of the probe which have both the dyes (one on each tail) and areas within the tails which are complementary to each other. The result is that the two tails of the molecular beacon will stick together and in so doing hold the reporter and quencher dyes in close proximity such that the quencher will prevent the fluorescence of the reporter. When the molecular beacon hybridizes into the target sequence the dyes will achieve a spatial separation which removes the quenching effect on the reporter and allows it to fluoresce. Quantitation can be achieved by measuring the fluorescent intensity, greater fluorescent intensity is shown when more target DNA is present. This proportionality between fluorescent intensity and target DNA concentration is identical to that seen with the TaqMan probe. Through spatial separation of the reporter dye from the quencher the quenching effect is removed and the reporter dye is made to fluoresce by exposing it to its specific excitatory wavelength of light in a microplate fluorescency reader. The only difference between the TaqMan assay and the Molecular Beacon is in how they achieve spatial separation of the two dyes, TaqMan does it by hydrolysis while Molecular Beacons do it by binding them farther apart on a single DNA strand. This technology has allowed the development of ultra high-speed real time PCR systems which is useful to disease control personnel (Belgrader *et al.*, 1999) to whom the ability to quickly identify disease outbreaks is an asset. This capacity is achieved by applying

a continuous flow PCR system with built in thermalcycling and product analysis into one portable machine.

Other detection systems have also been devised for reporting successful PCR amplification. One of these is an enzyme linked oligonucleotide probe (Deng *et al.*, 1996). In short, the detection system requires that the stop (antisense) primer be labeled with biotin at the 5' end and an oligonucleotide probe be linked to horseradish peroxidase (HRP). The stop primer labeled with biotin allows for binding of the target to the sides of a microplate well in solution. The HRP linked oligonucleotide probe is added to the binding solution and allowed to anneal to the single stranded target sequence. Any unbound substances are washed out of the microplate and then the HRP substrate solution is added. The HRP linked oligonucleotide will be retained if the biotin labeled primer is holding a target sequence in the well after washing. Once the substrate solution is added the HRP enzyme will catalyze hydrolysis of the substrate and a color change will become evident. The color change of that reaction can be analyzed using an ELISA reader to determine the presence or absence of target DNA (Deng *et al.*, 1996). In theory, this method can also be used to quantify the amount of target DNA present in the initial sample with the optical density being proportional to the amount of template in the original sample.

Technological advances have allowed for the production of very fast methods of thermal cycling, case in point is the continuous flow PCR chip (Kopp *et al.*, 1998). The traditional thermal cycler is eliminated with the continuous flow PCR chip in favor of a glass chip with a continuous channel arrangement cut into its surface. The reagents, as they flow through the channel, pass in and out of well-defined thermostatically controlled regions

heated by copper blocks. Continuous flow PCR is faster because the temperature changes of the reagents are virtually instantaneous. "According to Fick's law, the time needed for heat dissipation is directly proportional to the second power of the channel depth for a flat rectangular channel assuming that the thermostated copper blocks and chip represent an infinite heat capacity relative to the heated fluid element." (Kopp *et al.*, 1998).

1.6.1B Colony Hybridization Assay:

The colony hybridization assay involves the addition of a radio labeled oligonucleotide probe (specific for a target sequence in a gene of interest) to a hybridization solution. When the probe has had sufficient time to anneal to target DNA sequences the remaining unused probes are washed away. The actual process involves culturing samples on nitrocellulose filters (placed on agar), lysing the cells, denaturing the DNA and fixing it onto each of the filters through the use of a number of different solutions. The samples are ready for hybridization (addition of probe for annealing) when the unwanted cellular material is washed away (Ojeniyi *et al.*, 1994). The probes are radiolabeled oligonucleotide sequences specific for the target and successful hybridization is visualized through exposure to photographic film. Only specific target sequences can retain the probe and thus only the labeled samples can expose the film.

This brief review has demonstrated that many different modifications of the original PCR assay have been made. These new assays have greatly simplified the detection of specific DNA sequences in bacterial samples.

1.6.2 Immunologic Assays:

1.6.2A Enzyme Linked Immunosorbent Assay (ELISA):

The ELISA is an immunoassay technique based on the binding of monoclonal or polyclonal antibodies to a target antigen. Numerous formats of the ELISA have been utilized. One of these assays is a direct or non-competitive ELISA like the one used in this thesis. In this system, 96 well microtitre plates are first coated with a relevant antigen. One of five types of fimbriae [K88 (F4), K99 (F5), 987P (F6), F41, F18 (F107)] are suspended in a coating buffer and added to the wells of the plate to be incubated at 37°C for two hours. The excess material is then washed out and a blocking solution of irrelevant protein (5% w/v skim milk powder solution) is added to nonspecifically bind all of the unoccupied binding sights in the microtitre well. A test sample such as porcine serum can then be added which may or may not react with the bound fimbriae. If there is a positive reaction, the sample antibodies will be retained in the well during the washing while excess unbound material will be removed. After washing an immunoglobulin specific for the species of antibody to be tested can be added and allowed to react. That immunoglobulin is conjugated with some type of reporter molecule, typically the reporter is an enzyme conjugate that will catalyze a color or fluorescency change in a specific type of substrate molecule. The fluorescency or color change can be measured against a negative control to denote positive or negative results and against a positive control to quantify the results. The intensity of the color change is related to the activity of antibody present in the sample.

There are also variations on this theme. Competitive ELISA's compared to non-competitive assays test for the antigen by the same process of binding relevant antigens to the microtitre well. Once the plates are bound and blocked then a solution of both the test sample and the anti-fimbrial antibody specific for the target is added. If there is no target antigen present in the sample then all the antibody will adhere to the bound target antigen. However, if there is target antigen in the sample then some of the antibody will bind with it. In this way, the antibody remains in solution instead of binding to the well and will be removed during the washing step. At this point the conjugated anti-antibody immunoglobulin is added to the well and will react with the anti-fimbrial antibody that is bound onto the surface of the well. Again the substrate is added after the plate is washed and the reaction is observed. However in this case, the amount of reaction is inversely proportional to the amount of target fimbriae (more reaction, less fimbria in solution).

A further adaptation for ELISA technology is the sandwich assay for the presence of antigen (fimbriae). In this assay the primary antibodies are bound to the plate before blocking. Next the test sample is added and the target antigen reacts with the primary antibody and is retained through the subsequent washings. Next an anti-fimbrial antibody (secondary antibody) is added but it must be of a different species from the primary antibody to avoid nonspecific binding in the subsequent step. The excess is washed out after the secondary antibody has had time to interact with bound fimbriae. A conjugated anti-antibody immunoglobulin, termed the tertiary antibody, is then added to the reaction mixture. It reacts with the secondary antibody bound to the well by the antigen. This anti-antibody binding is species specific so the secondary antibody must be a different species from the primary otherwise the tertiary antibody conjugate would react non-specifically with

both the primary and secondary antibodies. The excess is washed out and the substrate is added to react with the enzyme conjugate. As in the non-competitive assay, the amount of reaction seen is proportional to the amount of fimbriae present in the original sample. (Coligan *et al.*, 1992)

1.6.2B Direct Immunofluorescent Filter Technique (DIFT):

The DIFT is an assay where sample cultures and suspensions are vacuum filtered through a polycarbonate membrane with an appropriate pore size (Fratmico and Strobaugh, 1998). Organisms that are captured on the membrane are exposed to an antibody specific for the target bacteria labeled perhaps with fluorescein isothiocyanate which will bind specifically to the organisms for which they are complementary (Fratmico and Terence 1998). Once the incubation is complete the membranes are washed to remove the unbound labeled antibody, then the remaining sample is examined with a fluorescence microscope; those membranes that hold cells that glow contain the target.

1.6.2C Antibody Direct Epifluorescence Filtration Technique:

This technique is a slight variation of the DIFT technique discussed above. In this method target cells are captured on a polyvinyl chloride (PVC) sheet coated with anti-*E. coli* antibodies. These sheets are incubated in a bacterial suspension and allowed to react with the potential targets. The sheets, when incubation is complete, are removed and lightly washed to remove bacterial cells that are loosely associated thereby leaving the tight antibody bound cells on the PVC. Next the sheets are incubated in a solution containing a fluorescein labeled anti-*E. coli* antibodies. If target bacteria are retained through washing by the first

antibody they will be bound by the labeled antibodies and thus be detected under fluorescent light. The PVC sheets can be observed directly using a light microscope equipped with a filter designed for fluorescence excitation. The captured bacteria can be concentrated through filtration. The cells are first scraped off the PVC sheets (5x6 cm) into a small volume of water that is then filtered through a black membrane filter sufficient to capture the bacterial cells (3 mm in diameter). The concentration factor is estimated to be about 424 times. The membrane filters are then observed with a fluorescence excitation microscope. Samples that fluoresce are considered positive for *E. coli* and those that do not are not (Wang and Sharp 1998).

1.6.2D Immunomagnetic Separation and Electrochemiluminescent Detection:

In this type of protocol a separation step is first used to concentrate the bacteria before analysis. The immunomagnetic-separation capture antibodies are prepared to specifically bind the target bacteria. The capture antibodies are biotinylated and mixed with streptavidin coated magnetic beads. The interaction between the biotin and streptavidin cause the binding of the capture antibody to the magnetic beads. The magnetic beads are coated with capture antibodies and when agitated in a mixed sample of bacteria and other substances will bind the target bacteria leaving the others in solution. A strong magnet is used to collect the magnetic beads some of which are holding the bacteria bound to the capture antibodies while those in the remaining solution are discarded. The beads can be washed as needed by suspending and separating the beads multiple times. The reporter antibody can be added once the washing steps are completed. A reporter antibody consists of an antibody of the same specificity as the capture antibody but with an

electrochemiluminescent heavy metal chelate like ruthenium(II) trisbipyridal chelate. This chelate functions as a reporter; a high-energy electron transfer excites it such that while the high-energy state is settling back to its ground state it emits a characteristic wavelength of light. In the case of the ruthenium(II) trisbipyridal chelate the light emitted during relaxation is at a wavelength of 620nm (Hao and Bruno, 1996).

1.6.2E Latex Bead Agglutination:

The basic concept of the latex bead agglutination test is that latex beads are bound to antibodies specific for a given target bacteria. Then the beads are exposed to a sample preparation and allowed to react with any target organism present resulting in agglutination of the latex beads. This is caused by cross-linking of the antibody receptors on different beads through multiple antigenic binding sights on one target organism. The end result should be a web-like mass of latex beads with antibody receptors bound to bacteria that are in turn are bound to other latex beads. There are many variants to this technique but the root concept remains the same, latex beads bound to antibodies are allowed to cross-link (agglutinate). The variation arises in the method of evaluating the agglutination. One such variation was explored by Arredondo *et al.* (2000) using a filtration flow-through apparatus. Detection involved the use of royal blue dyed latex beads with anti-*E. coli* antibodies attached. The first step was to add monoclonal IgM antibodies to a sample containing target *E. coli* bacteria that would begin the agglutination process by forming large immune complexes as is commonly seen with IgM antibodies. Shortly after the dyed beads with antibody were also added and then immediately filtered through a nitrocellulose membrane sheet. The pore size of the membrane was crucial to the assay, it needed to be large enough

so that the detection beads and IgM without target cells could pass through but small enough that the immune complexes would not be filtered out. Positive results showed a blue color in the membrane due to the dyed latex beads held in the immune complexes. There was no color in the membranes of samples that contain no target bacteria (Arredondo *et al.*, 2000).

1.6.2F Immunoblotting:

This procedure is also referred to as Western Blotting and is used to identify protein antigens using polyclonal or monoclonal antibodies. The protocol calls for separation of the sample proteins by SDS-PAGE a process whereby the sample proteins are solubilized in sodium dodecyl sulfate (SDS) and then separated by polyacrylamide gel electrophoresis (PAGE) according to their molecular weights. Once the gel has been run and separation is complete the gel is sandwiched between filter papers and spacer pads such that one side of the gel has a nitrocellulose membrane lining on it. The sandwich is supported in an apparatus that includes an electroblotting tank. Current is applied to electrophoretically draw the protein from the gel onto the nitrocellulose. The nitrocellulose is removed from the electroblotting apparatus, washed and the remaining binding sights are blocked. The first antibody specific for the antigen targeted is applied. The antibody that is nonspecifically bound is washed off and the conjugated secondary antibody (anti-antibody immunoglobulin specific for a given species) that will bind to first antibody is applied. The conjugate could be an enzyme that hydrolyzes a substrate to yield a color change reaction as seen with horseradish peroxidase (enzyme) and diaminobenzidine (substrate), (Coligan *et al.*, 1992).

In summary there are many different DNA and antibody based assays. The particular type of assay selected depends upon the needs and expertise of the analyst and the facilities that are available.

1.7 Treatment of Infected Pigs:

1.7.1 Conventional Treatment of Pigs for Diarrheal Diseases:

Pigs are treated on an individual or litter basis as needed when the incidence of diarrhea due to *E. coli* is low. Pigs at the University of Manitoba's Glenlea Swine Unit that are less than ten days of age are orally administered neomycin, sulphamethazine, and sulphathiazole in the form of Neorease™. They are then injected with Trivetrin™ containing trimethoprim and sulphadoxine. The animals must be treated therapeutically/prophylactically on a herd wide basis shortly after birth and perhaps even twice daily for several days if the outbreak is more widespread.

The routine use of antibiotics in farm animals is of considerable concern as the resistance factors in animal pathogens can be a detriment to sustainable use of antibiotics in animal agriculture. Sustainable alternative treatments must be developed as the widespread use of antibiotics in animals will undoubtedly be banned in North America as they have been in Europe. Concerned Veterinarians and Animal Scientists should ensure that antibiotics are properly used and that more environmentally friendly and sustainable alternatives are explored. Antisecretory drugs may be useful in the control of watery diarrhea and for the prevention further dehydration (Solis *et al.*, 1993). It should be noted, however, that this will

merely treat he symptoms not the disease itself. Oral electrolytes can also be particularly helpful in stemming the dehydration, ionic imbalance and maintaining body conditioning when diarrhea occurs in pigs over seven days of age (Gyles, 1994). Booster vaccinations of the pregnant females may be necessary if the problems are chronic. These vaccinations can include specific antigens or even diarrheic feces feedback as a form of an oral vaccine. Colostrum can be supplemented with high titer colostrum from specifically immunized sows or other forms of passive immune supplementation (Gyles, 1994).

1.7.2 Passive Antibody Treatments:

Passive antibody treatment is a highly effective method to control diarrhea (Yokoyama, *et al.* 1992; Owusu-Asiedu, 2002). Its mode of action is similar to that seen with colostrum. High titer antibodies are collected from animals that have been specifically immunized by receiving ETEC pathogen vaccine. The antibodies produced will be specific polyclonal antibodies against certain antigens such as K88 fimbriae. The antibodies, in the case of chickens that have been specifically immunized, can be obtained from the egg yolks of the layer hens, and then can be fed to young pigs in freeze-dried or spray-dried form in the feed to act as a prophylactic treatment against the specific ETEC. Specifically, in the work by Yokoyama *et al.* (1992) hens were immunized with preparations of fimbrial isolates from K88, K99 and 987P ETEC strains. The resulting antibodies from these hens specifically bound to the K88, K99, or 987P fimbrial adhesion particles, thus the binding of fimbria to the intestinal mucosa was decreased or inhibited depending on the dosage of antibody relative to amount of ETEC present in the small intestine. The ETEC were flushed out of the intestine with the fecal material. The antibodies thereby prevent

colonization and the production of large numbers of enterotoxin secreting bacteria that would lead to clinical ETEC diarrhea. This procedure can be used as a prophylactic or therapeutic treatment for ETEC diarrhea.

1.7.3 Dietary Antigen:

It has been theorized that isolated antigens such as K88 fimbriae when fed in the diet will bind to their receptor sites in the intestinal mucosa. In doing so, they effectively reduce or eliminate the binding of ETEC bacteria with the same fimbriae that would subsequently invade the intestine by occupying all the available binding sites. Consequently, the K88 ETEC cannot adhere to the intestine and are flushed out of the intestine with the fecal waste. This inhibition of colonization will prevent the large-scale replication of ETEC in the intestine, as a result there would be insufficient enterotoxin production to cause diarrhea. These antigens would be added to the feed for oral consumption by the animals. This could only be a prophylactic measure and would be ineffectual for treating existing cases of diarrhea.

1.7.4 Probiotics:

Probiotics work by inoculating the gut with so called positive bacteria. These are members of the normal flora which have no pathogenic effects and whose competitive effects are helpful in eliminating pathogenic organisms and therefore diarrhea (Walker and Duffy, 1998). Lactic acid bacteria are considered among the probiotic organisms and are used in the fermentation of yogurt among other things. The concept is that by inoculating these nonpathogenic bacteria into the gut they will occupy a niche in the intestine that will

exclude pathogenic organisms. Different modes of action for probiotic organisms have been proposed including the production of antibacterial agents specific for pathogens, stimulation of immune responses to suppress pathogens and competition for adhesion receptors and nutrients in the gut (Walker and Duffy, 1998). Probiotics are ususally fed orally for prophylaxis against pathogens as therapeutic effects probably would be minimal. The effects of probiotics can be difficult to predict and their efficacy can be variable or at times nonexistent (De-Cupere, 1992) with regards to oral *E. coli* challenge or changes in gut morphology (Nousiainen, 1991). Some researchers claim that probiotics when given to sows and their litters will increase feed conversion, daily weight gain, and total weight gain. It has also been said to decrease feed intake in pigs while maintaining weight gain targets and decreasing the prevalence of piglet diarrhea (Zani *et al.*, 1998).

1.7.5 Prebiotics:

Prebiotics are "nondigestible nutritional compounds that selectively stimulate the growth of endogenous lactic acid bacteria ... to improve the health of the host." (Walker and Duffy, 1998). Prebiotics are compounds that selectively promote the growth of favorable gut organisms; by feeding prebiotics some of the effects of probiotics are enhanced. Those effects may include receptor and nutritional competition as well as immune stimulation and the production of antibacterial compounds. Other beneficial effects thought to stem from the use of prebiotics are a reduction in blood lipids, enhance antitumor properties and hormonal regulation (Glenn, 1999). As a point of clarification, the designed mode of action for prebiotics is for them to act on the gut microflora instead of acting on the gut directly. The effects as seen with probiotics are not therapeutic, only

prophylactic. It has been observed that the effects of prebiotics are less than predictable and their effect on animal performance can be difficult to estimate (Mosenthin and Bauer, 2000).

1.7.6 Vaccination:

A vaccine is a preparation of compounds that will simulate infection by a specific disease while not actually being infective. Because the vaccine simulates the disease, the body initiates an immune response to fight the vaccine and thus prepares the body for the event of an actual infection. There are two ways of employing vaccines to protect piglets from ETEC. The first is to administer the vaccine to the sow before parity so the sow develops an immunity to the target organism to be passed on in the colostrum to piglets during nursing. This method provides passive immunity to the piglet that is effective for only a short time after weaning. Another method is to directly vaccinate the piglets which promotes the development of their own active immunity which in theory should last longer.

Varley *et al.* (1986) have studied the effects of oral vaccination on the survival and performance of early weaned piglets. It was thought that there would be some benefit in increased survival or weight gain but this was proven to be false as there was no increase in the survival rate of orally vaccinated pigs. There have also been some preliminary studies on the use of feeds producing artificial antigens that act as edible vaccines (Hugh *et al.*, 1998). In this study a transgenic plant was created to expresses the LT-B subunit. Mice fed this plant developed higher levels of serum and mucosal anti-LT-B antibodies than those given bacterial LT-B. These mice were then challenged with an oral dose of LT producing *E. coli.*

Though the mice were not completely protected they did receive protection that compared favorably with the bacterial vaccine (Hugh *et al.*, 1998).

According to Gyles (Gyles 1994) there are a number of commercially available vaccines for perenteral injection of pregnant sows and gilts made up of isolated fimbrial F4, F5, F6, F41 antigens and detoxified LT enterotoxin. The problem is that this would do little to supply a mucosal response directly in the gut. The majority of the response would be in the form of IgG whereas the most effective form is secretory IgA. For more effective protection of the animal an oral vaccine should produce a response that closely resembles an infection by the specific pathogenic ETEC. It is this type of vaccine that will yield an IgA response at the mucosal surface that lends a higher level of protection for sows and piglets who receive colostrum.

1.8 Conclusion:

Protecting pigs from ETEC diarrhea has been an area of great interest to researchers supplying products to hog producers and feed companies leading to the development of a number of strategies in an attempt to deal with the condition. The commonality between all these strategies seems to be the alteration of the microbial population in the gut of the animal. Pathogens can be targeted by narrow spectrum antibiotics and therapeutic antibodies or, more generally, with probiotics and wide spectrum antibiotics. The options open to producers may become far more limited in fairly short order with the withdrawal of antibiotics from use in animal agriculture forcing those in the animal industry to seek alternatives to suit their needs. Diarrheal disease control methods may initially be a matter

of producer preference based on the mode of action described by the distributor. However, the reputation of the product for effectiveness eventually will influence its competitive advantage in the market. It will not sell if it doesn't work! It becomes clear that the alternatives with a profitable future in the animal industry are those that are not only highly efficacious but are also safe, environmentally friendly, sustainable and competitively priced.

Objectives and Hypotheses

The objectives of this thesis are to apply the polymerase chain reaction to detect the presence of F4, F5, F6, F41 and F107 fimbrial colonization genes in pure strain bacterial cultures, mixed bacterial cultures inoculated with fecal swabs and fecal suspensions in water. Further objectives of this thesis are to apply enzyme linked immunosorbent and immunofluorescent assays to test for the presence of anti-ETEC fimbrial antibodies in pig serum samples and ETEC fimbrial antigens in bacterial cultures respectively. My hypothesis is that the PCR and ELISA methods can be applied as effective tools to test for the presence of enterotoxigenic *Escherichia coli* in the fecal swabs of pigs and for the presence of anti-ETEC antibodies in serum samples from the same pigs.

Optimization of the Polymerase Chain Reaction for the Detection of ETEC

2.1 Introduction

The PCR is a sensitive and specific means of detecting target bacteria based on their unique genetic character. In order to increase the efficiency of PCR reactions it is desirable to generate multiplex PCR's that test for more than one gene of interest at one time. In situations of multiplex PCR's that employ more than one primer pair in one reaction an interaction is sometimes seen between those primers and/or them and their targets, tentatively termed here as masking. Masking in PCR is the phenomenon where the activity of one primer set can cover that of another possibly due to an imbalance between those primers with regards to primer affinity and speed of hybridization to target sequences. The reason for the apparent masking or inhibition of primer activity could be accounted for simply by the dynamics of the PCR itself and the finite time available for each step of the reaction as controlled by the thermal cycler program. Specifically the time allowed for elongation can denote the amount of "enzyme time" each primed single stranded sequence receives with Taq pol. Consider a two gene multiplex PCR where two primer sets are at work in the same reaction vessel. Assume the activity of those two primer sets is equal when both are present at equal concentrations. The proportion of primed single stranded target sequences for both primer sets would be equal when the target DNA sequences for these primers are also present in equal concentration. In this case, the DNA polymerase enzyme (Taq pol) would perform its elongation function equally for both primed sequences and no masking would occur. However, the proportion of set A primed sequences to set B primed sequences would not be equal if the activity of primer set A greatly exceeded the activity of

primer set B. As a result primer set A would require a shorter hybridization time. Essentially primer set B would have to play "catch up" to build up an equivalent number of its primed sequences and to do that there is only a finite time available. When the concentration of primed sequences is not equal the amount of "enzyme time" allocated to each set primed segments would be proportional to the relative amounts of A primed segments and B primed segments ready for elongation. If the activity of primer set A is far greater than that of the B set then the number of B primed sequences becomes insignificant compared with the number of A primed. Subsequently, the amount of "enzyme time" B primed sequences receive becomes likewise insignificant compared to that of A primed sequences and thus PCR amplification products of B primed sequences become undetectable following electrophoretic separation. The objective of this study was to determine if masking of one fimbrial gene's amplification could occur when a multiple gene multiplex PCR was used.

In order to simulate increased primer activity the concentrations of the primers applied varied; since higher concentrations of primer simulate greater primer activity. A balanced bacterial standard for the testing will require that the two target gene sequences are present in equal proportions to ensure that any masking that may occur is not a result of a shortage of the target. The primers in a two-gene multiplex will be applied at a concentration where both produce satisfactory results in response to the 1:1 bacterial standard described above. This will establish a functional baseline. Once the activity of both of the primers is established at their baseline concentrations, primer A will be increased in concentration while that of primer B will be kept at the baseline concentration. Reduced activity of the B primer compared to that of A will be represented by a decrease in apparent PCR products

separated and stained by ethidium stain gel electrophoresis. Primer A then will be held at baseline and B will be increased until a similar point is reached. Studies such as these will provide guidelines on selecting and optimizing primer concentrations in multiplex combinations. Proper optimization of a multiple gene multiplex will help minimize false negative results.

This series of assays is intended to determine the relative masking effects of different primers for some of the common fimbrial genes. The primer pairs to be compared are F41 and F18, F41 and K88. Preliminary studies demonstrated that the F18 primers have the lowest activity and that the K88 primers have the highest activity (data not shown). Comparing them both to the F41 primer will illustrate the differences in activity of these primers relative to a common standard.

2.2 Materials and Methods

Standard bacterial samples (supplied by Dr. John Fairbrother and GREMIP, University of Montreal, Department of Veterinary Medicine, St Hyacinthe Quebec, Canada) were prepared for the PCR by inoculating pure cultures into Tryptic Soy Broth (TSB) and incubating overnight at 37°C. After incubation 20 ul of the culture was added to 500 ul of sterilized distilled water and kept in a boiling water bath for ten minutes (adapted from the protocol used by the aforementioned Dr. Fairbrother and the technicians in his lab).

The PCR reaction was carried out using the general procedure of Dr. John Fairbrother and GREMIP developed at the University of Montreal. PCR reaction volume

was 25 ul that was always mixed in pairs that were separated into individual reaction tubes. Each reaction mixture contained 2.5 ul of 2.5 mM dNTP, 2.5 ul of 10x Taq Buffer, 1.25 ul of each primer used in the multiplex, 0.2 ul of *Thermus aquaticus* derived DNA polymerase, and sterilized distilled water to a final volume of 20 ul. After the mix was made and distributed to the individual reaction tubes 5 ul of the bacterial sample preparation was added and the completed reaction mixture was centrifuged (10^4 g for five seconds) before thermal cycling. Concentrated (10x) Taq buffer was composed of 2.5 ml 1M KCl, 75 ul 1M $MgCl_2$, 500 ul 1M Tris buffer at pH 8.3, and 1925 ul sterilized distilled water. A 10x-dNTP mixture was composed of equal proportions each of 100mM dGTP, dCTP, dATP, and dTTP to yield a 25 mM dNTP mixture.

The reaction mixture was warmed in the thermal cycler (Techne Genius * Thermal Cycler for 96x0.2-ml microtubes) for 2 min at 50°C, and then the DNA was denatured from double stranded (dsDNA) to single stranded (ssDNA) by heating for 2 min at 94°C. A repetitious thermal cycle was run 30 times, each repeat consisted of 30 sec at 94°C to denature the DNA, 45 sec at 60°C to anneal the primers to the targets, and 40 sec at 72°C for elongation of the primed target sequence. Samples were held at 72°C for 10 min to complete the elongation of primed targets present. The samples were then kept at 4°C until removed from the cycler.

When thermal cycling was complete the samples were separated in a 1.8% agarose (Agarose A, Rose Scientific) gel stained with ethidium bromide (1 μg/ml). The agarose gel powder was mixed with TAE buffer and heated in the microwave for approximately 1.5 – 2.5 minutes until clear and free of bubbles. The gel, after the stain was added, was poured

into a mold and allowed to set. The mold defined a series of 60 wells at the top and middle of a 200 ml gel with a volume of approximately 15 ul. After the thermal cycle was complete 4 ul of loading buffer was added to the reaction mixture. This permitted visual determination of the distance of migration while electrophoresis was proceeding. The PCR products (15 ul) were loaded into each well for separation. These PCR products, as they migrated through the gel, were stained with the ethidium bromide. Higher concentrations of product in a given band within the gel were more heavily stained compared to bands having lower concentrations. The ethidium bromide stained gels were then exposed to ultraviolet light using a UV transilluminator (Ultra Lum U.V. Transilluminator model muvB-25 Ultralum, Inc.). The intensity of the fluorescent bands, based on visual assessment, were assigned scores between of 0 and 4+. A score of 0 represented a negative result, 1 was very weak, 2 was weak, 3 was good, 4 was very strong, and 4+ was excessive. The 50x TAE buffer consisted of 121 g Tris base, 28.5 ml glacial acetic acid, 50 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8 and 500 ml water. The gel loading buffer consisted of 25 mg bromophenol blue, 25 mg xylene cyanol and 4 g of sucrose in 10 ml of sterilized distilled water.

A balanced bacterial standard was constructed using pure cultures of target bacteria that were enumerated by separating the culture on Trypticase soy agar media. The cultures were diluted 390, 625:1 in 0.9% saline solution and 100 ul of the suspension was spread onto the surface of 4 tryptic soy agar plates with a glass rod. The plates were incubated overnight at 37°C and the number of colony forming units was counted while the original TSB cultures were refrigerated at 4°C to stop further culture growth. The number of colony forming units on the four TSA plates was averaged and used to calculate the concentration of the original

TSB cultures. Two pure strains of the cultures were then mixed in a ratio of 1:1. The standard was prepared for PCR by suspending 40 ul of the 1:1 bacterial mixture in 500 ul of sterilized distilled water and boiling it in a water bath for 10 minutes. The 1:1 bacterial standard (5 ul) was added to the reaction mixture and tested. The 1:1 bacterial mixture contained strains bearing the F18 and F41 or K88 and F41 fimbrial genes.

The University of Manitoba's Department of Microbiology synthesized the primers and their sequences used were as follows.

- K88 sense- aacctgcgacgtcaacaaga (Ojeniyi et al, 1994)
- K88 antisense- atcggtggtagtatcactgc (Ojeniyi et al, 1994)
- K99 sense- tgggactaccaatgcttctg (Ojeniyi et al, 1994)
- K99 antisense- tatccaccattagacggagc (Ojeniyi et al, 1994)
- 987P sense- tctgctcttaaagctactgg (Ojeniyi et al, 1994)
- 987P antisense- aactccaccgtttgtatcag (Ojeniyi et al, 1994)
- F18 sense- gtgaaaagactagtgtttatttc (Bosworth et al, 1998)
- F18 antisense- cttgtaagtaaccgcgtaagc (Bosworth et al, 1998)
- F41 sense- gagggactttcatcttttag (Ojeniyi et al, 1994)
- F41 antisense- agtccattccatttataggc (Ojeniyi et al, 1994)

The concentration of the stock solutions was calculated based on molecular weight of the primer and its optical density given that 1 OD=40 μg of DNA and the OD/ml was assessed by the manufacturer (University of Manitoba's Department of Microbiology).

Molecular weight of bases (Merck's Index):

Adenine = 135.14

Thymine = 126.11

Cytosine = 111.10

Guanine = 151.13

Molecular weight of primers =

$\Sigma[A(\text{mol. wt. adenine})+T(\text{mol. wt. thymine})+C(\text{mol. wt. cytosine})+G(\text{mol. wt. guanine})]$

$\Sigma[A(135.14)+T(126.11)+C(111.10)+G(151.13)]$

Where A= number of adenine bases in the primer, T= number of thymine bases, C= number of cytosine bases, and G= number of Guanine bases.

Concentration of primer:

1OD = 40 μg of DNA

x=OD/ml of primer

(xOD/ml)x(40 μg DNA)= (40xμgDNA/ml)x1000=40$x$pg DNA/ul = Concentration of stock primer solution

Working concentration of primer was 10pm/ul:

10pm/ul= 10x molecular weight of primer in pg = 10 mol. wt. of primer pg/ul

Dilution rate to working concentration:

(Conc. Stock Primer Solution pg/ul)/(Working Conc. Primer Solution pg/ul) = dilution rate

Based on the above calculations primers were diluted to a working concentration of 10 pmoles/ul as a base line and adjusted for the multiplex PCR as needed.

Table 2.1: Working Concentrations of Primers Used

Primer	OD/ml	Molecular Weight	Dilution Rate	Conc. (pmoles/ul)
1. K88s	84.00	2,648.40	126.87	10
2. K88a	67.00	2,604.46	102.90	10
3. F41s	98.00	2,638.39	148.58	10
4. F41a	113.00	2,567.36	176.06	10
5. 987Ps	112.00	2,583.35	173.42	10
6. 987Pa	83.00	2,552.35	130.08	10
7. F18s	121.00	3,058.82	158.23	10
8. F18a	102.00	2,752.54	148.23	10
9. K99s	74.00	2,608.37	113.48	10
10. K99a	59.00	2,586.40	91.25	10

2.3 Results

2.3.1 Baseline primer concentration for PCR assays:

The objectives of these studies were to determine the concentration of primers or combinations of primers (F18 and F41 or K88 and F41) that will yield a suitable reaction intensity when used in a multiplex assay. In these assays the concentrations of the ETEC's expressing the F18 and F41 or the K88 and F41 genes were constant in all assays. Several experiments were conducted but only the data from the final two experiments are summarized in tables 2.2 and 2.3.

The first study established the baseline concentrations of the F18 and F41 primer sets that were to detect their target gene sequences present concurrently in equal concentrations (Table 2.2). The F18 primer set when applied at a concentration of 30 μM yielded satisfactory positive results in a two-gene multiplex (F18 and F41) testing for one target strain (F18). This concentration of primers was considered to be its baseline concentration, therefore, no lower concentrations were subsequently used. The F41 primer set also showed excellent performance at 10μM concentration in a two-gene multiplex (F18 and F41) testing for one target strain (F41), therefore this was considered its baseline concentration. Normally the intensity of the electrophoretic bands obtained with 30 μM of F18 or 10 μM of F41 primer was between 3 and 4 when testing for the F18 or F41 target sequences alone. When the two-gene multiplex was applied to testing for both target strains at one time they also yielded similar electrophoresis results although F41 tended to have slightly stronger band intensities compared to that of F18.

Table 2.2: Baseline PCR Assay Results for F18 and F41

	Primer Concentration		Reaction Intensity	
Sample	F18	F41	F18	F41
1. $F18^+$	30μM	10μM	3	0
2. $F41^+$	30μM	10μM	0	4
3. $F18^+$=$F41^+$ (N=5)	30μM	10μM	3	4

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

The F18 and F41 primers were applied at their baseline concentrations to test a bacterial standard that contains both the target strains in equal concentrations.

The second study in this experiment established the baseline concentrations when F41 and K88 primer sets were used (Table 2.3). Baseline concentrations of the F41 and K88 primers were shown to be 20 μM and 10 μM respectively. At these concentrations both primers in monoplexes and two-gene multiplexes produced satisfactory individual and concurrent PCR product band intensities after electrophorestic separation. The resulting product band intensities of the single gene monoplex PCR's for K88 and F41 primers both had values of 4. In a two-gene multiplex testing for both K88 and F41 the K88 product band scored an intensity of 4 whereas the F41 primer scored only 3/4. The reasoning for this difference is unknown and may relate to the concentration of target DNA present in the two-gene multiplex standard relative to the monoplex standard.

Table 2.3: Baseline PCR Assay Results for K88 and F41.

	Primer Concentration		Average Reaction Intensity	
Sample	K88	F41	K88	F41
1. $K88^+$	10μM	20μM	4	0
2. $F41^+$	10μM	20μM	0	4
3. $K88^+$+$F41^+$	10μM	20μM	4	3

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

This assay set was designed to determine the baseline concentrations where K88 and F41 primers would produce satisfactory results in a two-gene multiplex for both genes.

2.3.2 F41 primer masking F18 primer activity:

The objective of this study was to determine if an imbalance in the concentrations of the F41 or F18 primers in a multiplex reaction would effect the amplification of the opposite gene in the reaction. In this study the concentration of the F41 primer was increased until a level was reached where F18 DNA amplification became depressed or undetectable when

the F18 primer concentration was kept at baseline (Fig 2.1 and Table 2.4). Both primers provided acceptable concurrent band intensities at their baseline concentrations. When the concentration of the F41 primer was increased to 20μM the amplification of the F18 was severely compromised yielding electrophoretic band intensities of 1/4. The intensity of the electrophoresis bands when the F41 primer was applied at concentrations of 30 and 40 μM was 4+, while the F18 band was undetectable. Therefore complete masking of the F18 gene occurred when the concentration of the F18 primer was held at 30 μM and that of the F41 primer was increased to 30 μM from a baseline concentration of 10 μM.

Table 2.4: F41 Vs F18 PCR Assay Results (figure 2.1)

	Primer Concentration		Average Reaction Intensity	
Sample	F18	F41	F18	F41
1. $F18^+$	30μM	10μM	3	0
2. $F41^+$	30μM	10μM	0	4
3. $F18^+$+$F41^+$ (N=5)	30μM	10μM	3	4
4. $F18^+$+$F41^+$ (N=5)	30μM	20μM	1	4
5. $F18^+$+$F41^+$ (N=5)	30μM	30μM	0	4+
6. $F18^+$+$F41^+$ (N=5)	30μM	40μM	0	4+

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

This is the assay set designed to test the F41 primer set concentration needed to mask the amplification of the F18 primer set.

2.3.3 F18 primer masking F41 primer activity:

Masking of the F41 primer was accomplished by holding the F41 primer at its baseline concentration of 10 μM and increasing the concentration of the F18 primer (Figure 2.2and Table 2.5). Previous assays demonstrated that at baseline concentrations both primers yielded satisfactory results. The band intensities for the F18 gene in the multiplex system containing both primers and both microorganisms increased dramatically with

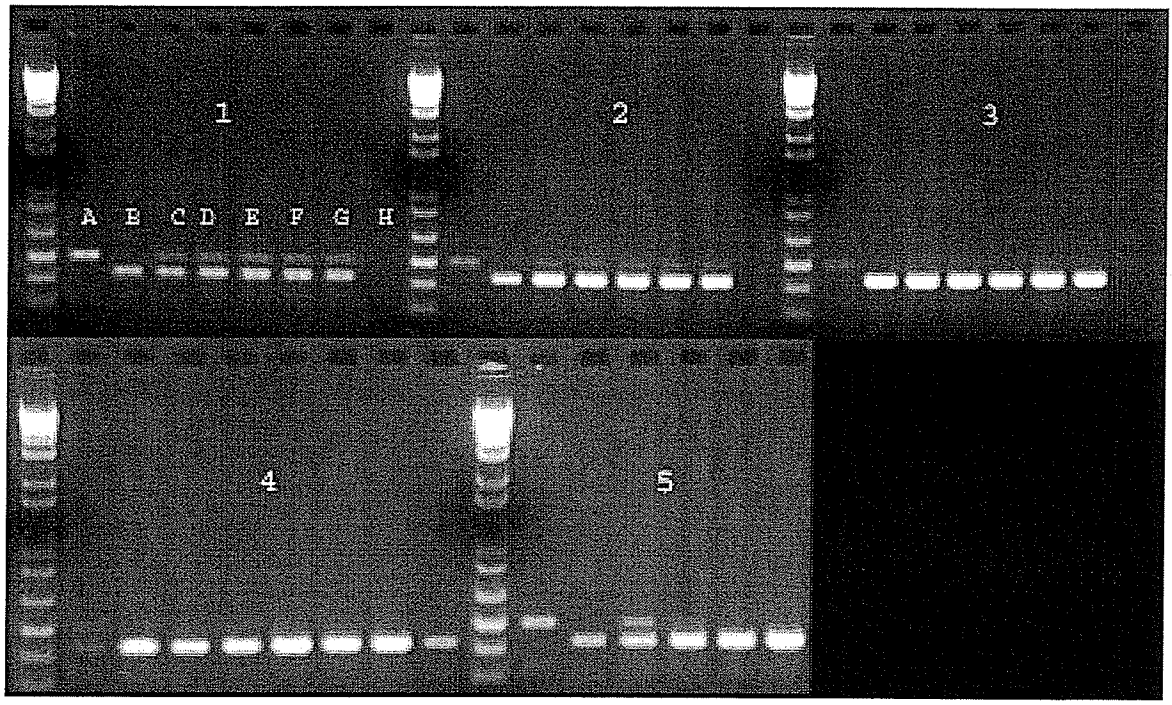

Figure 2.1: F41 Primer Masking F18 Primer Activity.

A typical electrophoretogram of a PCR reaction containing ETEC F18 and F41 with varying concentrations of the F41 primer with F18 primer concentration held constant. Lane A represents the F18 bacterial standard which separated at 510 bp as measured by the adjacent standard DNA ladder. Lane B represents the F41 bacterial standard whose amplified fraction separated at 430 bp. Lanes C through G represent the 1:1 $F18^+/F41^+$ bacterial multiplex standard and lane H represents the negative control. Primers in section 1 were applied at baseline concentrations of F18 at 30 μM and F41 at 10μM. The concentration of the F18 primer was maintained at 30 μM while the concentration of the F41 was increased from 10 μM in section 1 to 20 μM in section 2, 30 μM in section 3 and 40 μM in section 4. Total masking of the F18 primer was apparent at a concentration of 20 μM concentration of F41 primer. Section 5 represents all four previous sections with lane A showing the $F18^+$ bacterial standard, lane B showing the $F41^+$ bacterial standard and lanes C through F showing the effect of increasing F41 concentration on the reactivity of the F18 primer in a gradient-like fashion.

increasing concentration of F18 primer (from 30 to 80 μM). The corresponding intensity of the F41 gene at fixed primer concentration 10 μM decreased with increasing concentrations of F18 with the lowest value observed being 1.

Table 2.5: F18 Vs F41 PCR Assay Results (figure 2.2)

	Primer Concentration		Average Reaction Intensity	
Sample	F18	F41	F18	F41
1. $F18^+$	30μM	10μM	4	0
2. $F41^+$	30μM	10μM	0	4
3. $F18^+$+$F41^+$ (N=5)	30μM	10μM	1	4
4. $F18^+$+$F41^+$ (N=5)	40μM	10μM	3	4
5. $F18^+$+$F41^+$ (N=5)	60μM	10μM	4+	3
6. $F18^+$+$F41^+$ (N=5)	80μM	10μM	4+	1

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

This set of assays was designed to demonstrate the masking of F41 primer activity by increased concentrations of F18 primer.

2.3.4 F41 primer masking K88 primer activity:

Given the extremely high reactivity seen with the K88 primer it was expected that it would be difficult to demonstrate the masking of this primer with any of the others that were employed. The results confirmed these conclusions, as the band intensity of the K88 fimbrial gene was not greatly affected when the K88 primer concentration was held constant at 10 μM and the F41 concentration was increased from 20 to 100 μM (Figure 2.3 and Table 2.6).

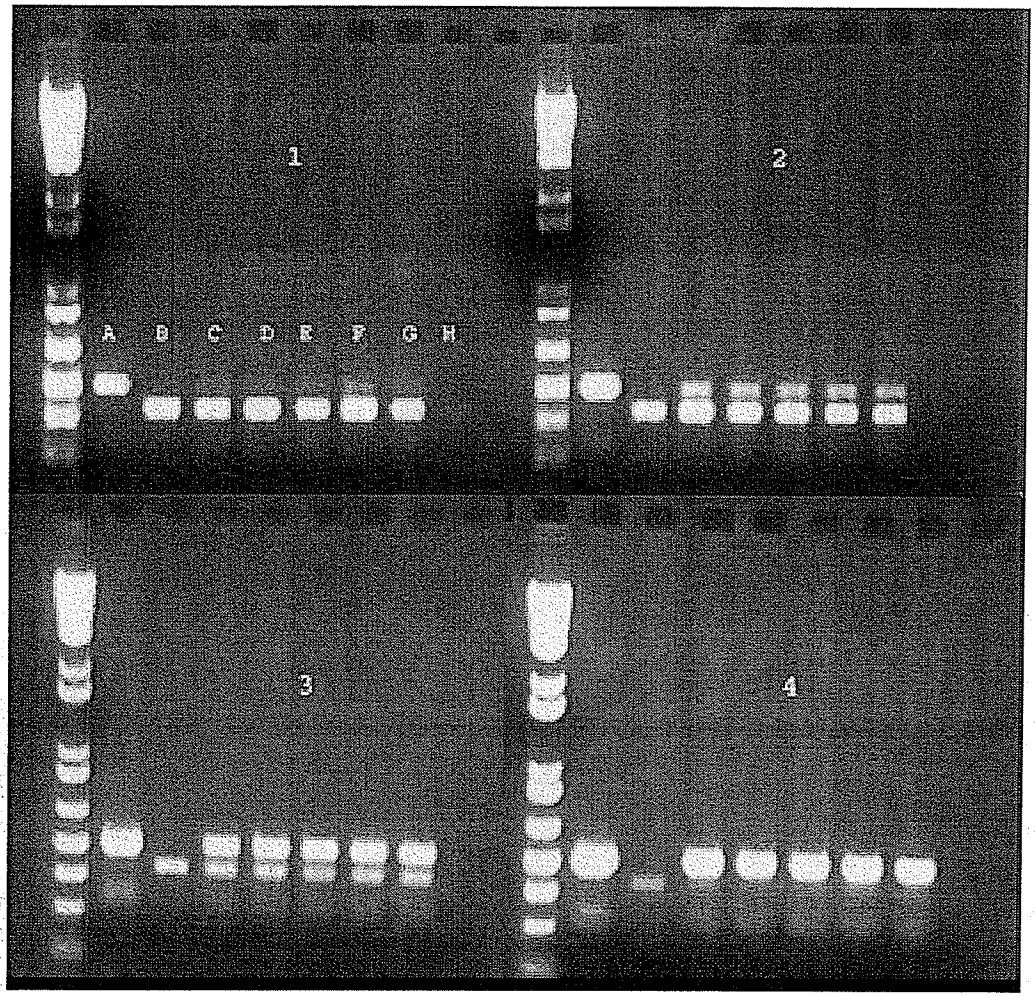

Figure 2.2: F18 Primer Masking F41 Primer Activity.

Lane A represents the F18 bacterial standard which separated at 510 bp as determined by the adjacent standard DNA ladder. Lane B represents the F41 bacterial standard which separated at 430 bp. Lanes C through G represent the 1:1 F18/F41 multiplex standard and lane H represents the negative control. The primers in section 1 were applied at baseline concentrations of 30 μM for F18 and 10μM for F41. The concentration of the F41 primer was maintained at 30 μM while the concentration of the F18 was increased from 30 μM in section 1 to 40 μM in section 2, 60 μM in section 3 and 80 μM in section 4. Masking of the F41 primer is most apparent at a concentration of 80 μM of F18 primer.

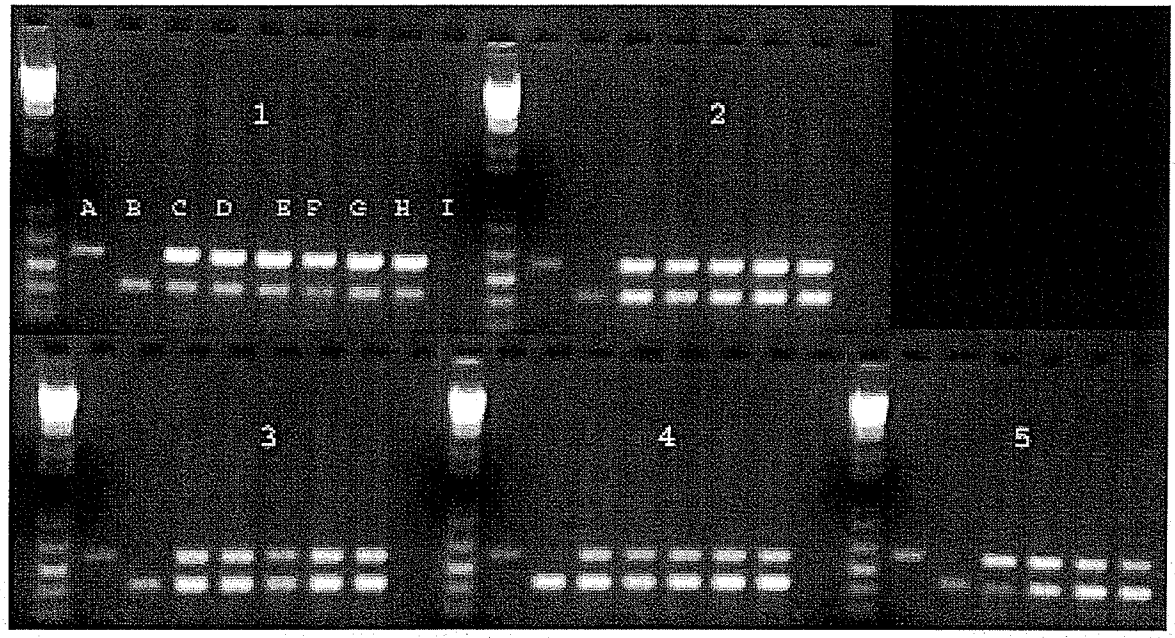

Figure 2.3: F41 Primer Masking K88 Primer Activity.

Lane A represents the K88 bacterial standard separating at 594 bp measured by the adjacent standard DNA ladder. Lane B represents the F41 bacterial standard that separated at 430 bp. Lanes C through H represent the 1:1 K88/F41 multiplex standard, and lane I represents the negative control. Section 1 applies the primers at baseline concentration of K88 at 10 μM and F41 at 20μM. The concentration of the K88 primer was maintained at 10 μM while the concentration of the F41 is increased from 20 μM in section 1 to 60 μM in section 2, 80 μM in section 3 and 100 μM in section 4. Total masking of the K88 primer was never complete but there was an apparent decrease in reactivity seen at 100 μM of F41 primer. Section 5 represents all four previous sections with lane A showing the K88 standard, lane B showing the F41 standard and lanes C through F showing the effect of increasing F41 concentration on the reactivity of the K88 primer in a gradient fashion.

Table 2.6: F41 Vs K88 PCR Assay Results (figure 2.3)

	Primer Concentration		Average Reaction Intensity	
Sample	K88	F41	K88	F41
1. $K88^+$	10μM	20μM	3	0
2. $F41^+$	10μM	20μM	0	4
3. $K88^+$=$F41^+$ N=5	10μM	20μM	4	3
4. $K88^+$=$F41^+$ N=5	10μM	60μM	4	4
5. $K88^+$=$F41^+$ N=5	10μM	80μM	4	4
6. $K88^+$=$F41^+$ N=5	10μM	100μM	3	4

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

This is the assay set to try to demonstrate the masking of F41 primer set over the K88 primer set.

2.3.5 K88 primer masking F41 primer activity:

Given the high activity of the K88 primer it predictably, should be easy for its activity to mask that of the F41 primer (fig 2.4 table 2.7). The baseline concentrations yielded band intensities of 3/4 for F41 and 4/4 for K88. The intensities of the corresponding F41 band decreased to an almost imperceptible level as the concentration of the K88 primer was increased from 10 μM to 40 μM. This shows the reciprocal relationship as seen in the F41 primer masking K88 experiments. The results demonstrate that K88 is a very strong primer that easily masks others while proving itself difficult to be masked.

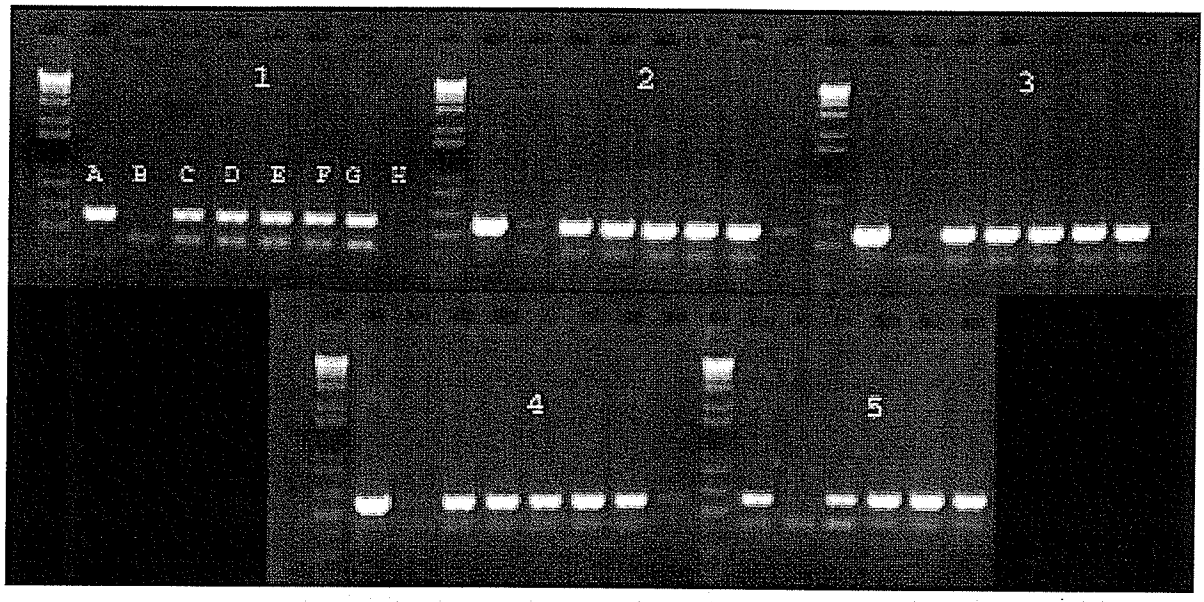

Figure 2.4:K88 Primer Masking F41 Primer Activity.

Lane A represents the K88 bacterial standard separating at 594 bp measured by the adjacent standard DNA ladder. Lane B represents the F41 bacterial standard that separated at 430 bp. Lanes C through G represent the 1:1 K88/F41 multiplex standard, and lane H represents the negative control. In section 1 the primers were applied at baseline concentrations, 10 μM for K88 and 20μM for F41. The concentration of the K88 primer was maintained at 10 μM while the concentration of the F41 was increased from 20 μM in section 1 to 60 μM in section 2, 80 μM in section 3 and 100 μM in section 4. Total masking of the K88 primer was never complete but there was an apparent decrease in reactivity seen at 100 μM of F41 primer. Section 5 represents all four previous sections with lane A showing the K88 standard, lane B showing the F41 standard and lanes C through F showing the effect of increasing F41 concentration on the reactivity of the K88 primer in a gradient fashion.

Table 2.7: K88 Vs F41 PCR Assay Results (figure 2.4)

	Primer Concentration		Average Reaction Intensity	
Sample	K88	F41	K88	F41
1. $K88^+$	10μM	20μM	4	0
2. $F41^+$	10μM	20μM	0	3
3. $K88^+$=$F41^+$ N=5	10μM	20μM	4	3
4. $K88^+$=$F41^+$ N=5	20μM	20μM	4	2
5. $K88^+$=$F41^+$ N=5	30μM	20μM	4+	2
6. $K88^+$=$F41^+$ N=5	40μM	20μM	4+	1

0=Negative, 1=Very Weak, 2=Weak, 3=Good, 4=Very Good, and 4+=Excessive.

This is the assay set designed to demonstrate the masking of the F41 primer by the K88 primer.

2.4 Discussion:

The results demonstrate that the outcome of a PCR reaction may be masked if the concentrations of the primers in a multiplex assay are not applied in the proper ratio. The reasons for masking may be explained as discussed in the introduction by an imbalance in the relative affinities of the primers for their target leading to an imbalance in the resulting products. If one primer has a significantly higher affinity for its target strand than others present in a multiplex it will bind to its target faster than its companion primers thus initiating the activity of the DNA polymerase on a larger scale during the finite annealing time. During elongation (the addition of complementary bases by DNA polymerase to a single stranded template) the proportion of target sequences duplicated will be identical to those primed. If the proportion of primed sequences for one target is far in excess of the others there will be little time left in the period of elongation where they can be replicated after the dominant proportion is completed. This example illustrates how high concentrations of one primed target strand can limit the time the DNA polymerase spends with the other primed target sequences. The concentration of high compared to the lower

affinity primer targets increases to a much greater degree as the 30x repeated thermal cycle continues thus perpetuating and magnifying the effect. This effect may have been responsible for the failure of the preliminary multiple gene multiplex assays (data not shown) as frequently only one target gene product would appear in an assay when two or three target bacteria were added. Increasing the concentrations of the other primers while keeping the reactive primer concentration constant had the effect of returning positive results for previously negative assays on identical culture preparations. In the light of these results it was necessary to optimize and standardize the baseline concentrations of the primers used in multiplex PCR's to ensure reliable results. The primer masking assays carried out in this study aided in this optimization as well as demonstrating the masking effect due to increased activity of one primer versus another.

The reasons for masking may have been multifactorial. The first and most obvious reason was the primer affinity, as discussed before which is related to the primary structure (sequence of nucleic acids in the primer) and possibly to the secondary structure that defines the interaction within the primer itself. The primary structure defines the ability of the primer to specifically and accurately hybridize onto the target sequence. The secondary structure defines the loops formed by internal complementarity within a single primer. It is thought that a secondary structure which shows a long 3' tail is desirable and that the length of the 3' tail may be proportional to the affinity of the primer for its target. Furthermore, the strength of the binding that forms the regions of internal complementarity is also thought to play a role as well as stronger bonds of internal complementarity are thought to decrease primer affinity. This pattern would seem to be followed by the K88, F41 and F18 primers (Table 2.8).

Table 2.8: SμMmary of Secondary Structure of Primers Used.

Primer Identity	Length of 3' Tail	Number of Internal Bonds	Tm of Internal Bonds	Optimized Baseline Concentration
K88 sense	3 bp	3 (1xAT, 2xGC)	10°C	10 μM
K88 antisense	8 bp	2 (1xAT, 1xGC)	6°C	10 μM
F41 sense	9 bp	2 (1xAT, 1xGC)	6°C	10-20 μM
F41 antisense	1 bp	2 (2xGC)	8°C	10-20 μM
F18 sense	0 bp	4 (3xAT, 1xGC)	10°C	30 μM
F18 antisense	1 bp	3 (2xAT, 1xGC)	8°C	30 μM
K99 sense	9 bp	3 (1xAT, 2xGC)	10°C	10 μM
K99 antisense	2 bp	3 (1xAT, 2xGC)	10°C	10 μM
987P sense	9 bp	3 (1xAT, 2xGC)	10°C	10 μM
987P antisense	8 bp	3 (2xAT, 1xGC)	8°C	10 μM

This figure represents the anticipated self-complementarity of the oligonucleotides examined.

Tm, as indicated in table 2.8 refers to the calculated theoretical temperature of melting at which primers anneal to their target, each guanine-cytosine bond adds 4°C to the annealing temperature while adenine-thymine bonds add 2°C. The primers were optimized as a pair meaning that the same concentration was used for both the sense and antisense primers, this may mean that either the sense or antisense primer may have a limiting effect on the pair. It should be noted that when the K88 sense and antisense primers are compared that the sense primer would appear to be limiting as it has a 3' tail only three base pairs (bp) long and a Tm of 10°C. The stronger antisense primer has an 8 bp 3' tail and a 6°C internal binding Tm. When in multiplex with F41 primers the K88 primer set always gives stronger product band intensities at baseline concentration. In the F41 primer set, the weaker antisense primer has a 1 bp 3' tail with an internal binding Tm of 8°C. The F41 sense primer has a 9 bp 3' tail and a 6°C internal binding Tm. Given that the F41 primer activity is a little weaker than the K88 it would seen that the length of the 3' tail plays a larger role than the strength of the internal binding Tm. The limiting K88 sense primer has a stronger internal binding with a longer

than the weak F41 antisense primer 3' tail. The strong F41 sense primer has the same internal binding strength as the strong K88 antisense primer and has a 3' tail one bp longer. Still the K88 primer set has higher activity, perhaps as a result of the longer 3' tail in the limiting primer.

There is an even sharper contrast between the design features of the F41 and F18 primers. With the F41 primer set there is at least one primer that could be considered well designed as far as the secondary structure and 3' tail length are concerned. In the F18 primer set both the sense and antisense primers have short or non-existent 3' tails while both show high internal binding Tm. According to the affinity theory it is no surprise that this primer set was optimized at 30 μM, the highest concentration of all the primers applied in this thesis. The compromised 3' tails would limit the ability of the primer to hybridize onto the target reducing the number of primed sequences at the time of elongation so fewer copies of that target would have been made. This pattern seems to hold true for all the primers used in this thesis, the K99 and 987P primer designs were very similar to the K88 and F41 primers and they both optimized at 10 μM.

However, the rationale behind the masking theory does not completely explain the results of the masking theory assays. Aside from the masking effect seen prominently in the multiple gene multiplex PCR's there was an effect seen in the single gene multiplex PCR's (those assays with multiple primers in the reaction mixture but with only one target bacteria added). It would appear that in the single gene standards there can be a slight decrease in the strength of the PCR product bands when the concentration of the competing primer is increased even though there is none of the competing primers target gene present. For

Table 2.9: Potential Strengths of Inter-Primer Binding Interaction.

Primers Interacting	Contiguous Matching bp's	MaximμM Matching bp's	Tm Contiguous Bonds	Tm MaximμM Bonds
1. K88s – K88a	3 bp	7 bp	10°C	22°C
2. K88s – F41s	3 bp	9 bp	8°C	28°C
3. K88s – F41a	3 bp	7 bp	6°C	20°C
4. K88a – F41s	4 bp	7 bp	10°C	20°C
5. K88a – F41a	3 bp	6 bp	10°C	18°C
6. F41s – F41a	5 bp	6 bp	16°C	18°C
7. F41s – F18s	6 bp	10 bp	14°C	24°C
8. F41s – F18a	4 bp	7 bp	10°C	20°C
9. F41a – F18s	4 bp	8 bp	12°C	20°C
10. F41a – F18a	3 bp	7 bp	6°C	18°C
11. F18s – F18a	3 bp	7 bp	6°C	18°C

Predicted interactions between primers with an oligonucleotide analysis program Oligotest V(2.0) – 1990, by C. Beroud.

complementarity that exist within the primer and define the length of the 3' tail that anneals to a target strand first. One of the theories that explain the occurrence of masking is based on self-complementarity, the shorter the 3' tail the lower affinity and annealing efficiency the primer has compared to primers with longer 3' tails. This theory expands to include the strength of the internal binding within a primer. With stronger internal binding regions primers will exhibit lower affinity and annealing efficiency. The primer efficiency and affinity will, therefore, effect the relative number of primed target sequences in a multiple gene multiplex and thus the number of sequences that would be elongated by DNA polymerase in the 40 seconds allowed in the thermal cycle program. The other theory is that the interaction between primers could account for the decrease of one primer's reactivity in the presence of another. A competing primer could prevent the action of a second primer if the two primers in a multiplex were undergoing significant binding to each other particularly if one of the primers was present in excess. At the same time the primer in excess would be

present in sufficient quantity to continue priming its own targets. The masking concentrations of two primers would, therefore, be identical if this were the sole cause of masking. This, however, was not the case for primers that showed strong reactivity. Decreasing their reactivity in the PCR required high concentrations of a competing primer while in turn the same results were seen with relatively low concentrations of the strong primer. This pattern of unequal masking by different concentrations of the primers more closely followed the Affinity and Efficiency theory suggesting that that is the dominant effect causing masking of one primer over another. However, there was another effect present too, one where the increased concentration of a competing primer decreased the reactivity of the single gene standard assay in a multiple gene multiplex (see Table 2.1 Lane A Sections 1-5). Given that there was no competition between primers for the elongation of more than one type of target DNA sequence, the interaction must have been between the primers themselves perhaps as a result of the binding of one primer to another. It should be noted that the decrease in single gene PCR products was not of the same magnitude as the masking seen in the multiple gene multiplexes testing for two targets simultaneously but they did decrease in a similar manner. Therefore, it would appear that the effects of masking seen in multiplex PCR's were a result of both interaction between the primers and within the primers.

Further studies are required to resolve some of the problems discussed above. This study also demonstrated that the multiple determination of gene products in a single PCR reaction must be carried out with caution so as to avoid false negative results. Nevertheless, even with it's problems the multiplex PCR reaction is a valuable tool that increases the

efficiency of diagnostic assays producing more data compared to monoplex PCR's using the same amount of reagents.

Clinical Trials

3.1 Introduction:

Two sets of animal trials were conducted in conjunction with *E. coli* challenge experiments carried out by Agustine Owusu-Asiedu (Ph.D. student at the University of Manitoba). In these trials 17 day-old pigs were orally inoculated with a predetermined strain of ETEC and so ideally the PCR should be able to detect this target organism in fecal swabs of inoculated animals.

The objective of the clinical animal trials was to demonstrate the use of the PCR to reliably detect the presence of target ETEC bacteria in fecal swabs of pigs. Since there was some difficulty in obtaining clinical diarrheal samples from animals environmentally challenged (infected by bacteria in their environment) artificially challenged pigs were the only realistic alternative. The added benefit of the artificial challenge was that the pigs were infected with a known strain of bacteria and so the bacteria shed should also be the same. In naturally challenged animals the infecting organism can be predicted based on trends related to the age of the animals and their receptors but there may be many other infectious and nutritional factors leading to piglet scours. In animals scouring from environmental conditions it is more difficult to estimate the numbers of false negative results for the PCR. With artificially challenged animals false negative results are easier to verify since all animals would have been inoculated.

3.2 Materials and Methods:

During the first trial piglets were received at ten days of age from Glenlea Swine Production facility. The piglets were orally inoculated with a 6-ml suspension of 10^{12} CFU $K88^+/F41^+$ *E. coli* bacteria in 0.9% saline at 17d of age (Owusu-Asiedu et al. 2002). Four hours after the pigs were inoculated they were checked for scours and rectal swabs of a random selection of piglets were taken. Samples labeled D1 I through D1 IV were inoculated into Tryptic Soy Broth culture mediμM for overnight enrichment at 37°C. Swabs were taken at four hours, 48 h, and seven days post inoculation. Four hours after inoculation the piglets were swabbed again and tested for the presence of the bacterial target. In the second animal trial, baseline or pre-inoculation swabs were taken from 10d old piglets and these were analyzed by PCR (Owusu-Asiedu et al. 2002). Four hours after the inoculation the piglets were swabbed again and tested for the bacterial target.

The bacterial inoculant was prepared by first culturing the bacteria in 500 ml of tryptic soy broth. After overnight incubation 1 ml of the TSB culture was spread on petri plates with blood agar mediμM. Petri plates were incubated overnight at 37°C and the resulting bacterial carpet was harvested using glass spreading rods to lift the bacteria from the agar into 0.9% sterilized saline. Piglets were given a 5-6ml oral dose of the suspension representing 10^{12} CFU. Further details on the experimental procedure can be obtained from the PhD Thesis of Mr. A. Owusu-Asiedu.

Bacterial samples were prepared for the PCR by inoculating fecal swabs into Tryptic Soy Broth (TSB) followed by overnight incubation at 37°C. After incubation, 20 ul of the culture was added to 500 ul of sterilized distilled water and kept in a boiling water bath for

ten minutes. The PCR was carried out as a K88/F41 two gene multiplex as outlined previously (page 35-36, this thesis).

3.3 Results:

PCR analysis of the inoculum cultured for both experiments demonstrated that it was a transgenic strain bearing genes for both K88 and F41 fimbria. It was established as a transgenic before the first animal trial when efforts to separate the mixed culture into two pure cultures with the agar streak method failed. Separation of the culture was attempted by streaking a loop of bacteria over an entire TSA plate and isolating single colonies from the streak for re-culture in TSB. This agar streak method was repeated a total of five times and the TSB re-cultures were tested each time for $F41^+$ and $F18^+$ genetic character. Each attempt at isolation yielded a broth culture with both $F41^+$ and $F18^+$ genes and so the strain was considered a pure transgenic.

There were no pre inoculation swabs taken from the first animal trial however the swabs taken after the inoculation indicate detectable levels of the K88/F41 ETEC in 80-85% of animals tested. A sμMmary of the results from the first clinical animal trial is outlined in table 3.1.

In the second animal trial during the pre-inoculation screening, six of the swabs were positive for F41 bearing ETEC although none of the piglets exhibited visible signs of scouring or tested positive for K88 ETEC. These swabs were a random sampling and no

identification numbers were recorded but the pen numbers of the six with detectable F41 were 25, 10, 27, 30, 11, and 15. On 19/5/01 (9 days before inculation) three clinical cases of scours were found and swabs of these animals were taken and their identification numbers were #129 (diet E6), #170 (diet E6), and #121 (diet E4). Of the three piglets only #129 proved to have detectable levels of F41 ETEC. Shortly after the swabs were taken the scouring resolved spontaneously. On 28/5/01 the piglets were swabbed again just before they were inoculated with approximately 10^{12} CFU of the target bacteria. Only three out of a total of 29 swabs from diets E1, E2, and E5 had detectable levels of F41 bacteria. The results of this trial demonstrated that prior to inoculation some of the pigs were positive for F41 but not for K88 ETEC whereas after inoculation with the transgenic ETEC 80% or more yielded swabs that were positive for the Transgenic ETEC (table 3.2).

Table 3.1: PCR Results for the First Animal Trial (11/01/01 – 18/01/01).

	K88	F41
Four Hours Post Inoculation		
Number Tested Positive	16/20 (80%)	16/20 (80%)
Average Band Intensity	3.8/4	3.8/4
Four Days Post Inoculation		
Number Tested Positive	17/20 (85%)	17/20 (85%)
Average Band Intensity	2.6/4	2.6/4
Seven Days Post Inoculation		
Number Tested Positive	16/20 (80%)	16/20 (80%)
Average Band Intensity	3.1/4	3.1/4

0=Negative, 1=Very Weak Band Intensity, 2=Weak Band Intensity, 3=Good Band Intensity, 4=Very Good Band Intensity. Pigs were inoculated on 11/01/01.

Table 3.2: Results of the Second Clinical Animal Trial 17/05/01 – 28/05/01.

	K88	F41
10 Day Old Baseline (none inoculated 17/05/01)		
Number Tested Positive	0/32 (0%)	6/32 (19%)
Average Band Intensity	0/4	2.3/4
Pre-inoculation (none inoculated 28/05/01)		
Number Tested Positive	0/29 (0%)	3/29 (10%)
Average Band Intensity	0/4	2.3/4
Four Hours Post Inoculation (all inoculated 28/05/01)		
Number Tested Positive	43/56 (77%)	43/56 (77%)
Average Band Intensity	3.8/4	3.0/4

0=Negative, 1=Very Weak Band Intensity, 2=Weak Band Intensity, 3=Good Band Intensity, 4=Very Good Band Intensity.

3.4 Discussion:

Based on the results of the animal trials two things are clear. The first is that the PCR can be used to identify the presence of target bacterial pathogens in enrichment cultures inoculated with fecal swabs taken from pigs environmentally challenged (infected by organisms present in their environment) as in the baseline assays of the second animal trial. Second is that pigs artificially challenged with a predetermined transgenic strain of ETEC have detectable levels of target ETEC in enrichment cultures of their fecal swabs. The transgenic strain represents an ETEC that bears both K88 and F41 fimbrial genes and so the simultaneous detection of both genes in the PCR should correlate perfect which was seen in both the clinical animal trials. This provides further confirmation of the reliability of the assay since transgenic strains of ETEC are not common.

There was, however, a discrepancy of 15 to 23 percent between the number of animals inoculated and those with fecal swabs that tested positive for the target pathogen. Some of the samples that tested negative after inoculation were potentially false negatives. The negative results could be verified by separating the enrichment culture on agar and selecting individual colonies for re-culture in broth. The process may be aided by separating the enrichment culture on blood agar that reveals hemolytic colonies which are more likely to be pathogens than the non-hemolytic colonies. This separation could compensate for the overgrowth of natural flora over that of the pathogen in the enrichment culture. The normal flora in the gut function as a non-specific host defense by occupying a niche in the intestinal environment and competing for available nutrients to the exclusion of pathogens. In some cases this defense is insufficient and the pathogens can colonize the gut causing scours if their populations are dense enough to produce high levels of enterotoxins. Even when the pathogens are present the normal flora persist and will grow in the enrichment culture. If the ratio of pathogens to normal flora is low enough, (if the normal flora are present far in excess of the pathogens) the normal flora could flourish to such an extent that they consμMe almost all the nutrients available as they would in the gut. This would competitively exclude the growth of the pathogen in the culture. However, fewer organisms are included in the mixed culture when individual colonies are selected and, as a result the target bacteria would be easier to detect with PCR. Another reason that could account for the unexpected presence of negative results may be that the treatment (therapeutic antibody inclusion in feed), as part of the challenge experiment, provided such an effective defense that the pathogen was never detected.

The presence of potential false negative results in the clinical animal trials may in part, be attributed to the utilization of available nutrients by normal flora in a mixed culture containing a pathogen to the exclusion of the pathogen. Another possibility is that non-target DNA can interfere with primer-target acquisition. The presence of large excesses of non-target DNA from the natural flora may effectively dilute target sequence DNA making it harder for the primer to "find" it and thus anneal to it.

3.5 Conclusion:

In conclusion, the PCR used in this study can be used to detect target pathogens in enrichment TSB cultures inoculated with fecal swabs from animals both environmentally and artificially challenged with ETEC. In these assays, false negatives may have occurred and are likely due to some factor related to the presence of non-target organisms or the effectiveness of the treatment included as part of the trial. Streaking the swabs or the enrichment cultures on blood agar and isolating individual colonies for re-culture in nutrient broth could be used to verify the negative results observed.

Direct PCR

4.1 Introduction:

Direct PCR is the direct testing of fecal swab suspensions for the presence of target bacterial DNA with no enrichment. The advantage of direct swabbing is that considerable time may be saved if enrichment procedures can be eliminated. Secondly the assay could be carried out on swab that do not contain live bacteria.

This trial was run in conjunction with the second animal trial and its objective was to determine if it is possible to detect orally inoculated target ETEC bacteria in the fecal swabs of pigs without enriching the samples before assaying. Directly tested samples were also compared to enriched samples taken from the same animals and in the event of a contradiction between the results the enriched sample results were considered to be correct.

4.2 Materials and Methods:

Five preliminary assays were carried out on 25/04/01 to determine if it was possible to directly detect target DNA in fecal suspensions. This preliminary assay was done as a three-gene multiplex testing for K88, F41, and 987P ETEC (which was the standard test at the time) although only K88 was shown to be positive.

Piglets were received at ten days of age on 17/5/01 for clinical trial 2 and on that date the baseline or pre-inoculation swabs were taken and analyzed by PCR directly and after enrichment on 18/5/01. All samples were taken in duplicate, one was tested directly and the

other tested after enrichment. Piglets were then orally inoculated at 17 days of age with a transgenic strain of ETEC that were positive for both K88 and F41 fimbrial genes. Fecal swabs were then taken four hours after inoculation in duplicate from these animals and tested for the presence of the ETEC inoculum. The bacterial inoculant was prepared using the same method as used in the first animal trial (page 58 this thesis).

Sample swabs were obtained from the University of Manitoba Animal Science Research Unit, in conjunction with an ETEC challenge experiment implemented by Augustine Owusu-Asiedu, Ph.D. student in the Department. Given the transgenic nature of the strain of ETEC it should be possible to detect both genes simultaneously in a two-gene multiplex PCR.

Standard bacterial samples were prepared for the PCR by inoculating pure cultures into Tryptic Soy Broth (TSB) and then incubating overnight at 37°C. After incubation 20 µl of the culture was added to 500 µl of sterilized distilled water and kept in a boiling water bath for 10 min. Mixed cultures from fecal swabs were prepared in the same manner with the exception that fecal swabs were used to inoculate the TSB instead of pure cultures.

Direct assay samples were prepared by suspending the fecal material of the swab in 2 ml of sterilized distilled water. In an effort to eliminate soluble PCR inhibitors the fecal suspensions were centrifuged (5 min at 8000 g) and the supernatants discarded. Pellets were re-suspended in a volµMe of sterilized water equal to one-half of the discarded supernatant then boiled for 10 min before testing with PCR in the usual manner (described on pages 35-36 this thesis).

Direct samples were tested using enriched assay results as a positive control, results of the comparison between the two assay methods were analyzed using a x^2 test with 1 degree of freedom. Comparisons were made between K88 positive results of the direct and enriched assays, F41 positive results of the direct and enriched assays and the K88 and F41 positive results of the direct assays.

4.3 Results:

The piglets used for this trial were the same ones used in the second animal trial described in the clinical trial chapter. All swabs from that trial were taken in duplicate and one of the swabs was reserved for direct testing. For more details see pages 59-60 this thesis.

A sμMmary of direct and indirect PCR assays of swabs taken from naturally challenged pigs in the preliminary examination (25/4/01) are presented in table 4.1. The results suggest that there was a reasonably good match between the two sets of results but that there were some discrepancies.

Table 4.1: Preliminary PCR Assays for K88 Fimbrial Genes in Fecal Swabs of Naturally Challenged Animals (25/04/01).

Sample Identification #	Intensity of $K88^+$ PCR Product Bands	
	Direct Results	Enriched Results
1. #407	4	2
2. #355	4	4
3. #357	0	2
4. #403	4	4
5. #404	0	0

0=Negaitve, 1=Very Weak Reactivity, 2=Weak Reactivity, 3=Good Reactivity, 4=Very Good Reactivity.

The following tables sμMmarize data from clinical animal trial two for fecal swabs analyzed by the direct PCR (Table 4.2) and those that have undergone enrichment (Table 4.3). The two sets of results were different as K88 and F41 ETEC were not detected in swabs taken from pre-inoculated pigs when directly tested (Table 4.2) but F41 ETEC was detected in the corresponding enrichment cultures. The swabs for the post inoculated pigs also yielded a different pattern of ETEC. The number of positive samples detected using the enrichment procedure was high (77%) and identical for K88 and F41 whereas both values were not the same and were lower when the direct assay was used.

Table 4.2: PCR Testing of Fecal Swabs Directly for the Presence of K88 and F41 Fimbrial Genes in Animal Trial Two.

	K88	F41
10 Day Old Pigs (none inoculated 17/05/01)		
Number Tested Positive	0/32 (0%)	0/32 (0%)
Average Band Intensity	0/4	0/4
Pre-inoculation (none inoculated 28/05/01)		
Number Tested Positive	0/29 (0%)	0/29 (0%)
Average Band Intensity	0/4	0/4
Four Hour Post Inoculation		
Number Tested Positive	36/54 (67%)	8/54 (15%)
Average Band Intensity	2.5/4	1.3/4

0=Negaitve, 1=Very Weak Reactivity, 2=Weak Reactivity, 3=Good Reactivity, 4=Very Good Reactivity.

Table 4.3: PCR Testing of Enrichment Cultures Inoculated with Fecal Swabs for the Presence of K88 and F41 Fimbrial Genes in Animal Trial Two.

	K88	F41
10 Day Old Pigs (none inoculated 17/05/01)		
Number Tested Positive	0/32 (0%)	6/32 (19%)
Average Band Intensity	0/4	2.3/4
Pre-inoculation (none inoculated 28/05/01)		
Number Tested Positive	0/29 (0%)	3/29 (10%)
Average Band Intensity	0/4	2.3/4
Four Hours Post Inoculation (all inoculated 28/05/01)		
Number Tested Positive	43/56 (77%)	43/56 (77%)
Average Band Intensity	3.8/4	3.0/4

0=Negaitve, 1=Very Weak Reactivity, 2=Weak Reactivity, 3=Good Reactivity, 4=Very Good Reactivity.

In the direct assays 15% of animals tested positive for F41 whereas 67% tested positive for K88 representing a statistically significant difference ($x^2_{1df, \alpha 0.05}=3.84<30.07$, reject H_o that positive results for both assays are equal in number). K88 positive results in the direct assays did not differ significantly compared to the K88 positive results of the enriched assays assays ($x^2_{1df, \alpha 0.05}=3.84>2.31$, accept H_o that positive results for both assays are equal in number). The comparison of the F41 direct and enriched assays yielded a significant difference in results($x^2_{1df, \alpha 0.05}=3.84<45.51$, reject H_o that F41 positive results of both assays are equal). False negative results were more prevalent in the direct assays for F41 as compared to the enriched assays indicating that enrichment helps lower the detection limit of target bacteria in the original swab sample.

4.4 Discussion:

The results of the direct assays taken from clinical trial two indicated that PCR values for K88 for the direct and indirect assay were in fairly close agreement. There may have

been some false positives obtained in the direct assay 4 h post inoculation since the results of the direct assay don't correlate with the enriched results. The intensity of the PCR product bands for F41, however, were much lower in the direct compared to the indirect assays. The percentage of swabs that tested positive for both K88 and F41 was much higher in swabs that were from enriched compared to those analyzed directly.

The fecal suspensions from the five preliminary swabs were both tested directly and enriched in TSB. Cultures that enriched the direct assay suspensions should show perfect correlation with the direct PCR assay results if the direct assay results are indeed accurate. Unfortunately the comparison of the direct assay results does not correlate perfectly. In the direct assay samples #407 and #355 proved positive with very good reactivity while #357 showed no reaction. All the samples showed positive reactivity after enrichment but the reactivity of the #357 and #407 samples were weaker than the direct assays. The appearance of an apparent false negative in the direct testing of the fecal swab suggests that the requisite number of target bacteria needed for a successful reaction was not present and that enrichment may indeed be a necessary step in the diagnosis of ETEC causing diarrhea in swine. The only way to determine how essential enrichment is to PCR detection is to conduct a similar set of assays on a far larger test group under more controlled conditions. Only then will the proportion of false negative results be clear.

Based on the larger sample size in the clinical animal trials it is clearly possible to detect target pathogen DNA in the fecal material of animals orally inoculated with a high concentration of a known ETEC strain. In the current study the ETEC was a $K88^+/F41^+$ transgenic strain bearing both fimbrial genes. Four hours after inoculation fecal swabs were

taken and tested, the target strain was detectable. However, the efficiency of the K88 primer set is high and it's detection limit is apparently lower than that of the F41 primer used.

The preliminary findings would suggest that it is possible to directly test for the presence of K88 (F4) ETEC without enriching the fecal flora in culture media both in animals naturally and artificially challenged but the results are not as reliable as those of the enriched samples. However, the activity shown by the K88 primer set employed was remarkably high and may have played a role in the results of the preliminary findings. Other primers used here are not as highly reactive and so their powers of direct detection are not as great. This effect was discussed previously. Based on the assays performed in this study it is concluded that direct testing for the presence of target ETEC is possible but the results are not as reliable as those of the enriched samples are.

Factors contributing to the unreliability of direct testing could prove to be many and varied. The most obvious would seem to a concentration of the targeted DNA sequences that is below the detection limits of the protocol used n this study. There are large populations of symbiotic bacteria in the intestine of the host animal that are part of the beneficial normal flora. The normal flora are able to suppress the growth of the pathogenic target bacteria in the animal by consμMing available nutrients to the exclusion of target pathogens. When the mixed culture, as represented by the fecal swab, is inoculated into TSB all of the normal flora may not be nutritionally supported as they are in the gut. This would allow the pathogen to flourish in the culture media relative to the normal microflora, a condition that does not occur in the intestine. Another factor that could influence the detection of the target pathogen is interference with the assay itself by conditions intrinsic to

the fecal suspensions themselves. There may be substances in the fecal material that are heat stable (for 10 min in a boiling water bath) and water insoluble (found in the precipitate from centrifugation) that inhibit the Taq Pol enzyme. The presence of a large amount of insoluble material in the fecal suspension could also dilute the concentration of target DNA present in the sample. When the samples are held in the boiling water bath all the bacterial cells present are ruptured and as such there are undoubtedly large amount of non-target DNA present in the sample that may also interfere with the amplification of the target when present. One or all of these factors could inhibit or interfere with a successful PCR reaction leading to the production of false negative results that would not seen in the enriched sample assays.

4.5 Conclusion:

In conclusion, it is possible, however unreliable, to detect target pathogens in fecal material suspended in water with this PCR protocol. The unreliability of the direct assay may stem from the presence of insoluble materials in the feces inhibiting the DNA polymerase enzyme. The presence of non-target DNA from the natural flora may also interfere with the acquisition of the primer for the target. A combination of both factors coupled with the lower number of bacteria present in the direct assays as compared to the enriched samples could possibly account for the production of false negative results. Furthermore, there is a larger component of preparation required for direct assays increasing the labor required for routine testing. Currently, given the unreliability and increased work required for sample preparation, direct PCR testing for the presence of target pathogens does not appear to be practical.

Enzyme Linked Immunosorbent and Immunofluorescent Assays.

5.1 General Introduction:

Enzyme linked immunosorbent and immunofluorescent assays (ELISA and ELIFA) were originally to be used in this thesis as a cross-reference technique for the PCR. The ELISA would have been used for the testing of pig serμM samples for the presence of antibodies against target antigenic molecules, specifically the enterotoxigenic *E. coli* (ETEC) fimbrial adhesins F4, F5, F6, F41 and F107. The ELIFA was to be employed in testing for the presence of these fimbrial adhesins directly in the enrichment cultures inoculated by fecal swabs taken from scouring pigs. These two techniques together with the PCR would have been used to conduct a survey of Manitoban hog barns for the presence and prevalence of these five strains of ETEC and how they are regionally and seasonally distributed. In this thesis all but the antibody ELISA were examined but only in preliminary tests and were not studied in depth or optimized for use in routine testing.

5.2 Introduction to the Non-Competitive Antibody ELISA:

The non-competitive ELISA system is useful for the detection of target antibodies and works by exposing samples of unknown antibody titer to desired target molecules bound to a solid phase media. In this study isolated fimbria from F4, F5, F6, F41 and F107 ETEC were bound to the inner surface of wells in a 96 well microtiter assay plate. Antibody samples were then processed as necessary, added to the wells of the microtiter assay plate, and incubated. Antibodies that were complementary to the bound fimbria (termed the

primary antibody) were held by these fimbria through subsequent washing steps that removed all unbound antibodies. The bound antibodies were then exposed to a secondary antibody, a species-specific immunoglobulin that was conjugated with a phosphatase enzyme. The second antibodies bound by the first were also retained in the plate through subsequent washing steps assuring the enzyme was present for the addition of the phosphatase substrate solution. The enzyme conjugate indicator catalyzed the hydrolysis of a phosphatase substrate to yield a color change. The intensity of the color change was quantified by its increase in absorbency (optical density) compared to a blank well using an ELISA reader. There was a proportional relationship between absorbency and antibody titer; therefore, the greater the absorbency, the higher the antibody titer of the sample. A survey for the presence of antibodies in the pig serμM specific for the target ETEC fimbria would indicate previous exposure of an animal to a given strain of ETEC. This would indicate a chronic infectious state in the animal or perhaps provide a pattern of infection in a production facility.

5.3 Materials and Methods of the Noncompetitive ELISA:

5.3.1 Coating Plates with Antigen:

Source of anti-K88 antibodies:

Anti-K88 rabbit serμM antibodies and affinity purified anti-K88 chicken antibodies were prepared in the laboratory of Dr. R.R. Marquardt following the standard procedure

outlined by Kim et. al. 1999. Anti-K88 partially purified anti K88 chicken egg yolk antibodies were prepared as outlined by Clarke et. al. 1993.

Testing for antibody requires that the target antigen for which the antibodies are specific must be bound to a solid phase media. Transparent 96 well microtiter assay plates (Falcon 3912, Microtest III flexible assay plate, 96 flat bottom wells) were used as the solid phase media. Isolated fimbrial antigens were diluted in a 0.05 M carbonate-bicarbonate buffer solution (pH 9.6) (Carbonate-bicarbonate buffer capsules, Sigma cat # C-3041), to a concentration of 10 μg/ml. Next, 100 μl of the fimbrial solution was added to the wells of the assay plate to coat each of them with 1 μg of fimbrial isolate. Assay plates were incubated at 37°C for 2 h after which they were washed and blotted 5 or 6 times with a washing solution of PBS-T (0.5 ml Tween 20/l of PBS). The plate wells were each filled with the PBS-T and left for 3 min. The solution was shaken out and the plate was blotted with paper a towel. After the coating process was completed the binding sites of the microtiter assay plate wells were blocked. A solution of irrelevant protein, 5% skim milk powder solution, was added at 200 μl per well to block all non specific binding sights not occupied by the fimbrial isolates. PVC plastic plates will bind proteins indiscriminately; the blocking solution precludes binding of the antibodies to all but the fimbrial isolates. Plates were incubated for 2 h at 37°C then subsequently washed using the same method described previously.

Concentrated PBS (phosphate buffered saline 20x) was prepared as follows. 4.6 g NaH_2PO_4 (5.29 g $NaH_2PO_4 \cdot H_2O$), 23 g Na_2HPO_4, 180 g NaCl, all dissolved in 1000 ml of water. The solution was to be adjusted to pH 7.2-7.4 with NaOH or HCl.

5.3.2 Antibody ELISA:

After the transparent immunoassay plates were coated with one of F4, F5, F6, F41 or F107 fimbrial isolates they were loaded with the antibody samples. First all the wells on the assay plate were filled with 100 µl of PBS, and then the lead wells (A1-A12) of each colµMn were filled with 100 µl of the antibody samples to be tested. Two colµMns were reserved for the positive control (antibody standard with an arbitrary antibody titer of 1.0). The standard antibody had to be from the same species as the unknown samples because the secondary antibody was specific for the species of antibody being tested. The other lead wells were filled in pairs (two replicates) with the samples to be tested. They were mixed and 100 µl from the lead wells was successively transferred to the next well in the colµMn creating a 2x serial dilution along the colµMn. The last well in each colµMn was not loaded with antibody as it served as the negative control. The plates were incubated for 2 h at 37°C during which time the antibodies interacted with the bound fimbria. The antibody solution was then shaken out and washed with PBS-T as in the coating procedure. After washing was complete the second antibody was added to react with the first antibody held by the bound fimbria. The second antibody was an affinity-purified immunoglobulin specific for the Fc region of the species from which the antibody originated. The secondary anti-antibody immunoglobulin was conjugated with a phosphatase enzyme, it was diluted 3000:1 in PBS, then 100 µl of that solution was added to each well on the microtiter assay plate. Once loaded with the conjugated immunoglobulin solution the plate was incubated for 1.5 hours at 37°C. After incubation was complete the plates were washed as before and 100 µl of the

substrate solution was added (2x Sigma 104 Phosphatase Substrate Tablets in 10% diethanolamine 90% 0.5mM $MgCl_2$ buffer). The reaction between the enzyme and the substrate was allowed to proceed for 15-20 min at room temperature before the resulting absorbency of the color change was measured with an ELISA reader (Bio Rad Model 3550 microplate reader with Microplate Manager version 4.0 software) using a 405 nm filter. Absorbency of the color change was proportionate to the titer and concentration of the antibody solution added. The titer of the antibodies was calculated based on the comparison of two curves, the first was from the serial dilution of the positive control standard with an assμMed relative titer of 1.0. The other curve was that of the serial dilution of the unknown antibody sample. The titer of the antibody sample was calculated as a proportion of the standard curve with a titer of 1.0. The antibody titer is simply the dilution rate of the antibody sample at the given absorbency value (usually at a midpoint of the curve) divided by the dilution rate of the standard antibody with the same absorbency value.

5.4 Results and Discussion of the Noncompetitive ELISA:

The noncompetitive ELISA in my studies most extensively tested the titer of chicken egg yolk antibodies (see figure 5.1 for a typical assay curve). The yolks were diluted 1:2000 in PBS before being added to the lead wells of the assay plate. A few preliminary assays were also performed on pig serμM antibodies and seemed to work best when diluted 1:25 in PBS. Assays of this sort had been used and proven by researchers in the past. In this light it seemed unnecessary to reprove work that had already been done. Since no more work needed to be done on them they were ready to be employed in the proposed survey of Manitoban hog barns. At that time the main focus of this thesis was to be the survey and not

on the development and optimization of assays. However, the development of the fluorescency assay for antigens and the adaptation of the PCR took much longer than expected leaving no time for the survey to be initiated much less completed. Since the survey was not initiated the pig serμM antibody assays were not followed up or tested more rigorously.

5.5 General Introduction to Sandwich and Competitive ELIFA for Target Antigens:

The sandwich enzyme linked immunofluorescent assay (ELIFA) system was intended to be used to detect the presence of F4, F5, F6, F41 and F107 target fimbriae in the enrichment cultures inoculated with fecal swabs from scouring pigs. A fluorescency system was used in an effort to increase the sensitivity of the assay over and above that seen with the absorbency based ELISA system. Molecular Probes, Inc. claim that their fluorescein diphosphate fluorescent phosphatase substrate can lower the detection limits fifty fold as compared to conventional absorbency substrates. Two types of ELIFA systems were attempted in this study for the detection of target fimbria; the first was a sandwich ELIFA and the second on competitive ELIFA. The first assay involved the binding of rabbit antibodies specific for fimbriae to a solid phase in the reaction vessel followed by exposure to bacteria producing the targeted fimbriae. In theory, the fimbrial particles bind to the first (primary) antibody and are held through the washing steps for presentation to the secondary antibody. The primary rabbit antibody and the secondary chicken antibody are both specific for the same target bacterial fimbriae and together they form the top and bottom layers, sandwiching the antigen between them. Next a third (tertiary) antibody is added, with the same phosphatase-conjugated immunoglobulin specific for chicken species antibodies that

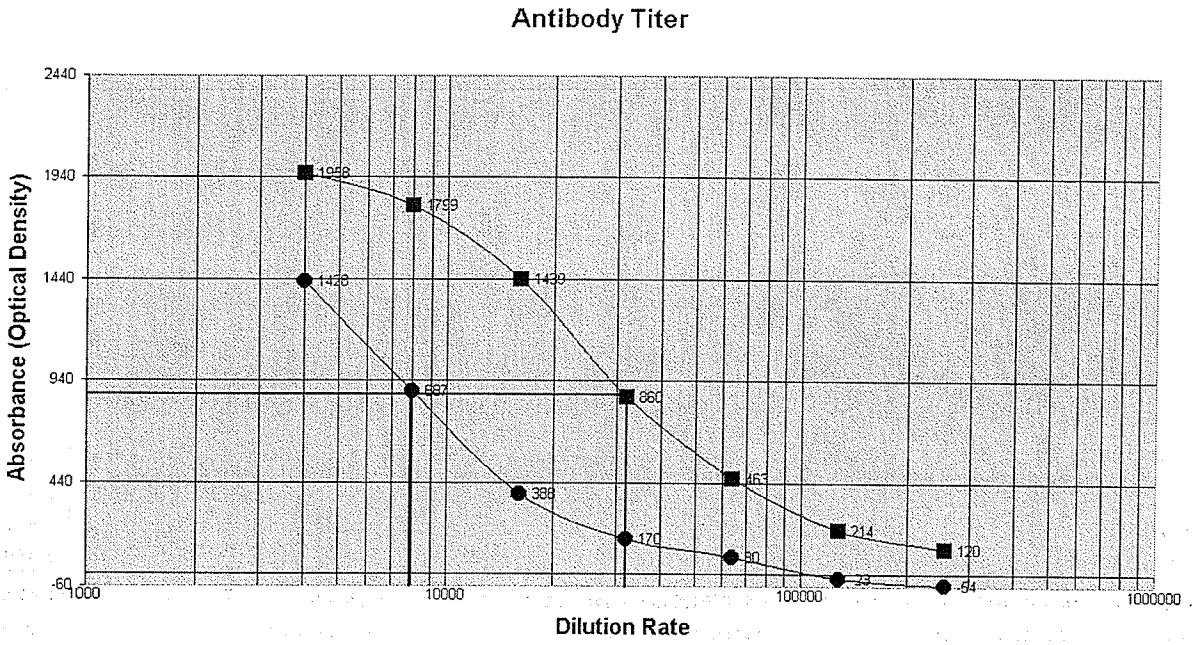

Figure 5.1: Typical Antibody Titer Graph.

Determination using a non-competitive ELIFA, of the anti-antibody titer in an unknown (●) and a standard reference sample (■). Each well was coated with K88 fimbriae from ETEC. The curves were produced by the serial dilutions of the two samples. To calculate the titer of the antibody sample a line was drawn through midpoints of both curves corresponding to an absorbency value of 0.85. The dilution of the standard curve where it intersects with the line is 32000:1. The dilution of the antibody sample where it intersects the line is 8000:1. The relative titer value of the antibody sample is calculated to be 0.25 when the midpoint dilution of the antibody sample (8000:1) divided by the midpoint dilution of the standard antibody curve (32000:1) bearing the same absorbency value. The samples were analyzed in duplicate. See Materials and Methods for further details.

was used in the antibody ELISA discussed previously. Binding of the secondary antibody to the tertiary antibody holds the tertiary through the subsequent washing that removes the unbound tertiary antibody conjugate. The phosphatase enzyme catalyzes the hydrolysis of the substrate with the addition of a fluorescent substrate solution. Substrate hydrolysis yields a change in its fluorescent character that can be quantified with a fluorescency reader. The intensity of the change in fluorescency is proportional to the concentration of fimbria present in the sample.

The second type of assay was the competitive assay where target fimbria, both bound and in solution, compete for the same binding sights on the soluble antibodies. When fimbria are present in solution with complementary antibodies they will decrease the binding of antibodies to the bound target fimbriae. The concentration of soluble fimbriae, (they represents the sample being tested) is inversely proportionate to the amount of complementary antibody that will bind the immobilized fimbriae. The competitive ELIFA, being highly sensitive, was to detect levels of antigen that the sandwich assays could not. In preliminary assays the sandwich assay could detect purified target antigens but it had little success in detecting antigens at the concentrations present in a typical TSB culture containing the target bacterial strain.

5.6 Materials and Methods for Sandwich ELIFA:

5.6.1 Coating Plates with Antibody:

Black opaque 96-well microtiter assay plates (Costar 3915, solid black opaque plate, 96 flat bottom wells, non tissue culture treated) were coated with 1.0 μg/well of rabbit anti-fimbrial antibodies (prepared in the laboratory of Dr. R.R. Marquardt according to protocol outlined by Kim *et al.* 1999) suspended in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Each of the wells on the microtiter assay plate were filled with 100 μl of the antibody solution and incubated at 37°C for 2 h. The plates were then washed and blotted 5 or 6 times with PBS-T as in the previous assays. After the coating process was completed the remaining binding sights of the microtiter assay plate wells were blocked with the same 5% skim milk powder solution that was used in the blocking of antigen coated plates discussed previously.

5.6.2 Sandwich ELIFA for Target Antigen:

The preliminary work for optimization of the assay was done using targeted fimbrial isolates at various concentrations ranging from 1 mg/μl down to 125 μg/μl. The isolates were prepared as outlined in the paper by Kim *et al.* (1999). Fimbrial isolates were diluted in PBS and added to the lead wells of each colμMn in plates coated with the rabbit antibody

followed by 2x serial dilutions as previously described. The plates were then incubated for 2 h at 37°C before washing. The rabbit antibodies coating the wells therefore held the fimbrial isolates in the wells after washing. Subsequently, 100µl of a $1:3x10^4$ diluted solution of affinity purified chicken antibodies (produced by the staff of University of Manitoba's department of Animal Science under the supervision of Dr. R.R. Marquardt) was added to each well on the assay plate and incubated for 2 hours at 37°C. After incubation the plate was washed and each well filled with 100 µl of a 3000:1 diluted phosphatase conjugated affinity pure rabbit anti-chicken IgY in PBS. The plates were then incubated for 1 h at 37°C, during which time the rabbit anti-chicken immunoglobulin conjugate binds with the chicken antibodies. In the final step the substrate solution was added. It was composed of a 30 µM solution of fluorescein diphosphate (Molecular Probes F-2999) in a 10% diethanolamine, 90% 0.5 mM $MgCl_2$ buffer (pH 7.2) termed the alkaline phosphatase buffer. The hydrolysis reaction catalyzed by the phosphatase enzyme was allowed to proceed for 20 min at room temperature. After that time the fluorescent intensity of the hydrolysis product was measured with a fluorescency reader (Spectramax Gemini by Molecular Devices operated by Softmax Pro Version 3.1.1 software, ex 490 nm, em 520 nm). The intensity of emission wavelength light was proportional to the amount of fimbrial isolates present in the sample. Therefore, the standard curve for fimbriae for a sandwich ELIFA would be similar in appearance to that of the ELISA curve.

5.7 Results and Discussion of the Sandwich ELIFA:

Preliminary studies demonstrated the sandwich ELIFA was not highly sensitive even though a fluorescent indicator was used. The lower limits of detection were somewhat lower than 0.375 or 0.75 mg fimbria/ml solution depending on whether the fimbrial preparation was or was not sonicated (figure 5.2). As indicated in the figure, the relative fluorescent intensity was considerably higher in sonicated compared to non-sonicated samples. This may be attributed to the fragmentation of the fimbrial subunits in the thread like pillus into smaller fragments each of which would contain several fimbrial subunits. This in effect would have increased the number of antibody binding sights with a given amount of fimbrial protein, thereby, enhancing the apparent amount of fimbria in the sample. Additional research needs to be carried out to determine if the sensitivity of the assay can be increased by further dissociation of the fimbria into its subunits. Other means of increasing sensitivity of the assay also needs to be explored. In addition there was considerable intra- and inter assay variability (data not shown). It is conceivable that the ELIFA assay for the K88 fimbrial antigen of ETEC is not suitable as used in this study, for the detection of this pathogen.

5.8 Introduction to the Competitive ELIFA for Detection of Target Antigen:

The second type of ELIFA applied for the detection of target fimbria was a competitive assay system. Since this was a fluorescency-based system, black opaque plates were used in place of the transparent plates. This competitive assay is initially identical to the non-competitive antibody ELISA in that target fimbria were bound to the solid phase media. A suspension of pure bacteria bearing the same target fimbria as were bound to the microtiter assay plate was then added to the wells followed by the addition of a suspension of

antibodies complementary for both the bound and the free antigens. The two sources of fimbria (bound and free) will bind the suspended antibody competitively in proportion to their concentrations. The antibodies bound to the soluble fimbria are removed during the washing steps and the rest are retained, bound to the microtiter assay plate by the fimbria attached to the solid phase media. The remaining bound antibodies are allowed to react with the phosphatase-conjugated IgY that are specific for species of animal antibodies to be tested. The same system was used in the ELISA for antibodies described above. Those antibodies that are bound are retained through the washing steps. The fluorescent substrate, fluorescein diphosphate (FDP), is added and the products of the hydrolysis reaction are measured using a fluorescency reader. Substrate hydrolysis is inversely proportionate to the concentration of target fimbria present in the sample tested.

5.9 Materials and Methods of the Competitive ELIFA:

5.9.1 Coating Plates with Antigen:

The procedure for the coating of plates with antigen in preparation for a competitive ELIFA was modified slightly compared to that used for the ELISA (see section 5.3.1). Essentially the only difference was that the transparent microtiter assay plates used in the antibody ELISA were substituted in the sandwich ELIFA with black opaque plates (see section 5.6.1).

5.9.2 Competitive ELIFA for Antigen:

The wells of the plate were filled with 100 µl of PBS and then 100 µl of the antigen suspension was added to the lead wells (A1-A12) mixed and transferred to the next well in the colµMn. This gave a 2x serial dilution along the colµMn that was repeated up to the last well that was reserved for negative control. The positive control standard was a solution of isolated target fimbria at a concentration of 200 ng/µl in PBS buffer. After the serial dilution was complete 100 µl of $1.5x10^4$ diluted affinity purified or $5x10^3$ diluted chloroform separated anti-K88 chicken antibodies was added to all the wells. The mixture was incubated at 37°C for 2 h, washed and blotted. During this time the bound and soluble

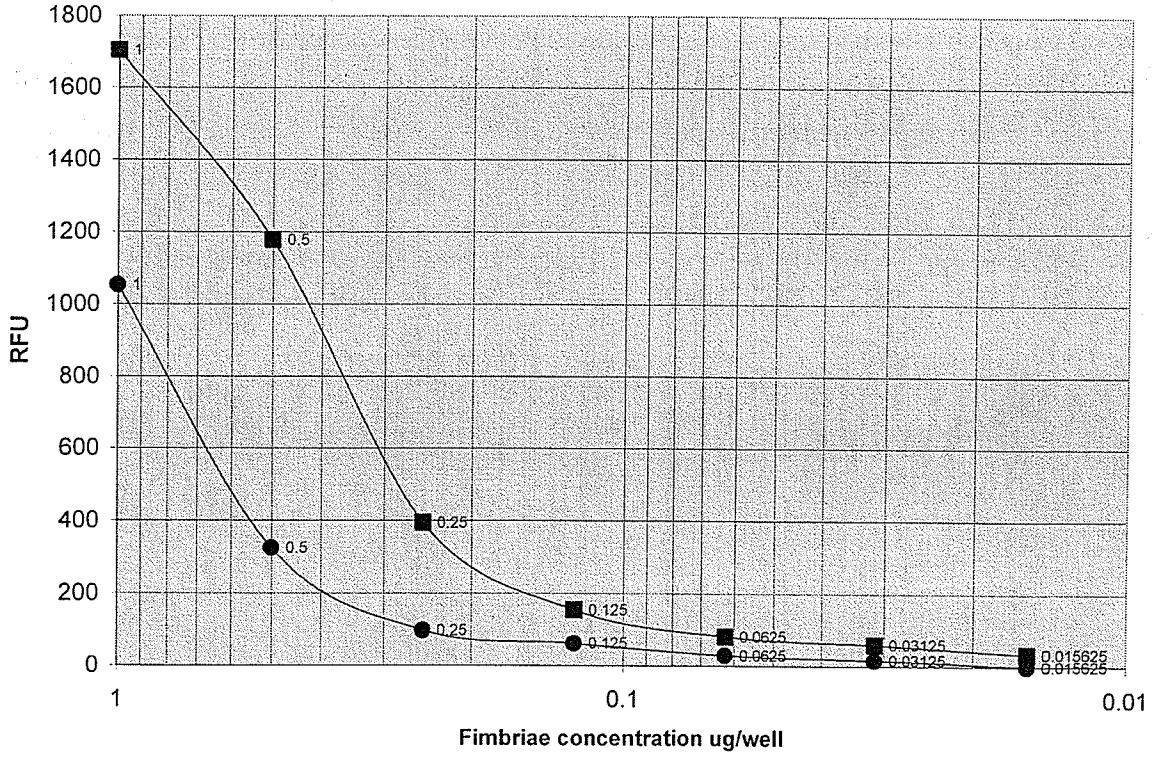

Figure 5.2: Typical Sandwich ELIFA for K88 Fimbria.

A typical sandwich ELIFA for K88 fimbria using rabbit anti-K88 ETEC fimbrial antibodies as the coating (primary) antibody. Different amounts of K88 fimbrial antigen were incubated with the rabbit antibodies and affinity purified chicken anti-K88 antibodies were used as the secondary antibody. This graph represents two curves generated during a sandwich ELIFA. The upper curve (■) shows the 2x serial dilution of a solution of K88 fimbrial isolate. The initial concentration of fimbriae in well 1 was 1 mg/ml suspended in PBS after 3 minutes of sonication. The lower curve (●) was generated by 2x serial dilution of the same 1 mg/ml solution of K88 fimbrial isolate but without the sonication. The relative concentrations detected with and without sonication at 50% of the maximμM absorbency were 0.375 mg/ml and 0.75 mg/ml respectively. The samples were analyzed in a Spectramax Gemini fluorescency Reader (Molecular Devices 1311 Orleans Ave. Sunnyvale CA 94089-1136 U.S.A.). See Materials and Methods for further details.

antigens competed for binding sights on the complementary anti-fimbrial antibodies. Antibodies that bound the soluble antigens were removed in the subsequent washing steps while the antibodies that bound to fixed antigens were retained through the washing steps. After incubation of the antibody/antigen solution was completed and washed out, the same affinity purified phosphatase conjugated rabbit anti-chicken IgY that was used in the antibody ELISA was added to the wells and incubated. This detected the presence of chicken antibodies that were bound by the target fimbria after 1.5 h, the time allowed for binding of the phosphatase-conjugated IgY to the antibodies. The plates were washed and blotted. Finally 200 µl of FDP phosphatase substrate was added to the all the wells of the plate and after a 20 min incubation at room temperature the hydrolysis of the substrate was quantitated with a fluorescency reader. As previously stated, the fluorescent intensity of the products was inversely proportionate to the concentration of fimbria present in the sample

5.9.3 Preparation of Chloroform-separated Chicken Egg Yolk Antibodies

The chloroform-separated antibodies were prepared following the method designed by Clarke et al (1993). In this procedure one volµMe of freeze-fried egg yolk was diluted in four volµMes of PBS and one volµMe of $CHCl_3$ (chloroform) followed by shaking for 5 minutes. The sample was centrifuged for 30 min at 10,000 xg and the supernatant was saved. Fourteen percent (w/v) of polyethylene glycol (PEG) 8000 was added to the supernatant, mixed well and stored overnight at 4°C. After standing overnight at 4°C centrifuge for 15 minutes at 10,000 xg and harvest the precipitate. The precipitate was re-suspended in PBS and the precipitation with PEG was repeated to further purify the antibodies otherwise. The pellet containing the purified antibodies was re-suspended in PBS

and freeze-dried. The freeze-dried antibodies were re-constituted by mixing in PBS at a concentration of 2 mg/ml.

5.10 Results and Discussion of the Competitive ELIFA:

The concentration of fimbria in a competitive assay is inversely proportional to the hydrolysis of the fluorescein diphosphate substrate by the phosphatase enzyme.
In this assay a dilution of 1:8 for the bacterial suspension yielded an RFU value similar to that obtained by a 1:128 dilution of the fimbrial standard (Fig 5.3). This means that the concentration of the K88 (F4) fimbriae was approximately 12.5 ng/ml (200[8/128]=12.5) (figure 5.3). The competitive assay can therefore be used to successfully test for the presence of the F4 fimbriae. The method also seems to be of higher sensitivity than the sandwich assay since 12.5 ng/ml of fimbria were readily detected with the limits of detection being below this value. However, the number of K88-ETEC that was equivalent to a given amount of K88 fimbriae was not established. This assay, used high titer, affinity purified chicken serμM antibodies which were available in limited quantities and there were no such antibodies available to expand the system to the other target fimbria. Chloroform separated chicken egg yolk antibodies (CSCA's) were used as a substitute with limited success. This assay, therefore, could not be extended beyond the preliminary test. With greater time and resources available affinity colμMns could have been constructed to isolate more monoclonal antibodies which would have been applied to the other four targeted ETEC strains. Because of the nature of the assay itself optimization is a matter of great importance and difficulty, the researcher needs to find a balance between too much antibody and too little. If too much antibody is used a situation of binding saturation occurs where the antibody is present in sufficient quantities to adhere to all the bound and soluble antigen in

all the wells. In this case no curve would arise and the assay would be useless. If there's to little antibody present then the detection limits of the antibody conjugate/substrate combination are insufficient to detect it. Likewise in this case no curve would result and the assay would be useless. The failure of this assay when using anything but affinity purified antibodies may have been due to the variability of antibody sources not purified in this way. Perhaps the activity of these types of antibodies was so variable that they could not be optimized to the fine point of balance reliably. This could have been a result of the activity of the antibody themselves or more likely the result of background interference from contaminants (fats and other proteins) in the antibodies applied. A further complicating point of this type of assay was the concentration of fimbria targeted which was very low to simulate the conditions of a typical bacterial culture. This would further complicate the delicate balance between binding saturation and undetectability required for the assay to work.

This system if more widely successful would have been able to test for the antigens present in acute cases of scours which would have given a good cross-reference to the PCR detection of the fimbrial gene.

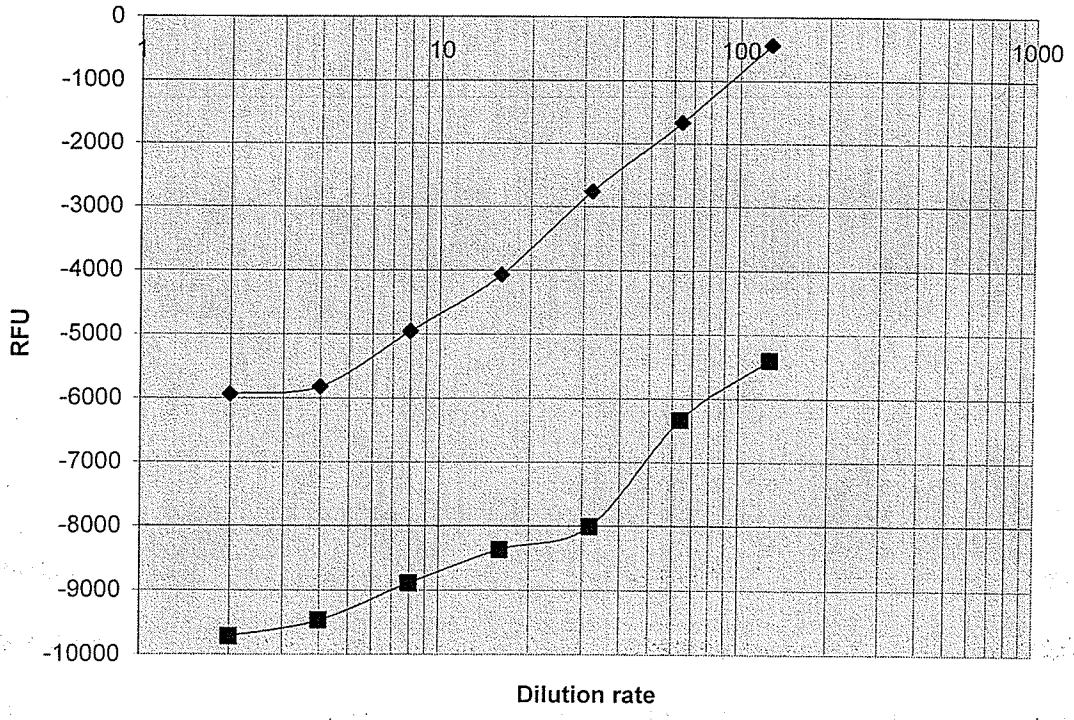

Figure 5.3: Competitive Assay for Fimbria.

This figure represents two curves resulting from the competitive assay for K88 fimbriae. The lower line (■) represents the curve for a 2x serial dilution of the positive control (a solution of K88 fimbrial isolates), with an initial concentration of 200 ng/ µl of PBS. The upper line (♦) is the line for a 2x serial dilution of K88 bacteria suspended in PBS (concentration of the bacteria unknown). In a competitive assay the concentration of fimbria present is inversely proportional to the hydrolysis of the fluorescein diphosphate (FDP) substrate by the phosphatase enzyme. The higher relative fluorescency units (RFU) of the bacterial sample compared to that of the standard indicates that the concentration of fimbria present in the bacterial sample is less than that of the standard. The approximate concentration of the bacterial sample is 6.0% of that of the control (200 µg/µl). This value was calculated by dividing the dilution rate of the sample by that of the standard at a given value of RFU. This gives the concentration of the sample as a proportion of the standard in this case the concentration of the fimbria in the unknown sample was 12.5 ng/µl. The samples were analyzed in duplicate. See Materials and Methods for further detail.

5.11 Discussion on ELIFA/ELISA:

Initially the focus of this thesis was to establish a pattern of both seasonal and regional distribution for the five strains of enterotoxigenic *Escherichia coli* causing scours in hog production facilities throughout the province of Manitoba. The primary method of detection was to be the polymerase chain reaction for the detection of ETEC in fecal swabs. That would be cross-referenced when necessary by an enzyme-linked immunofluorescent assay directly testing for the presence of target fimbriae in the same fecal swabs. These two assays of fecal swabs would determine if the cause of acute scouring in pigs were a result of one of K88, K99, F41, F18 or 987P enterotoxigenic *Escherichia coli.* In addition, an enzyme-linked immunosorbent assay for the presence of anti-ETEC antibodies would test for the presence of antibodies complementary for K88, K99, F41, F18 and 987P fimbria in serμM samples of pigs. The presence of these antibodies in the pig serμM would be an indicator of long term disease exposure that elicited an active immune response. These three assays, when used in conjunction would have provided a complete and comprehensive picture of the past and present disease status in a given production facility.

The sandwich enzyme-linked immunofluorescent assay for the detection of ETEC antigens was to be developed after the non-competitive antibody ELISA was mastered. The early stages of the sandwich ELIFA showed promise when they were used to test for the presence of pre-prepared fimbrial isolates from K88 ETEC bacteria. Levels tested were 125, 250, 500, and 1000 μg/μl, all of which showed smooth useful curves. Based on the initial work, a positive control standard was established using the isolated K88 fimbria at a

concentration of 200 μg/100 μl. The curve resulting from this assay could be used to calculate the concentration of the antigens in an unknown sample, as a percentage of the standard. The same method was used to calculate the titer of antibodies an ELISA. This assay could then provide an estimate of the number of ETEC in a sample if the concentration of the antigen/bacterial cell was known and constant.

Next some experiments were conducted to compare the binding activity of whole fimbrial isolates with those that had been sonicated for various times to break the fimbria into subunits to hopefully increasing their reactivity. Sonication did increase the reactivity as samples containing constant concentrations of fimbrial isolates showed higher RFU values after 3 min of sonication. However, even with sonication, the apparent detection limits of fimbria was only about 2 mg/ml.

It was hoped that the use of a fluorescent substrate would have decreased the detection limits thereby increasing the sensitivity of the assay (lower the amount of fimbria present needed for a positive result). According to the manufacturer (Molecular Probes) their fluorescein diphosphate (FDP) tetraammoniμM salt could provide a detection limit as much as 50 times lower than that obtained with the chromogenic substrate, *p*-nitrophenyl phosphate. It was for this reason that the conventional ELISA substrate was replaced with the fluorescency substrate in hopes that it would make it easier to detect small amounts of the antigens in a bacterial culture. There were two main problems evident in the sandwich ELIFA as it was being run at the time. The first being that the positive control which consisted of 2 mg/ml of fimbrial isolates typically gave stronger responses than that of the bacterial cultures tested which contained approximately 10^9 CFU/ml. This concentration is

about one million fold greater than the desired detection limits. The second problem was that the curves that did result from the bacterial cultures showed high variation and the portion of the curve representing the lowest four (out of eight) points returned values less than zero relative fluorescency units and so were useless. These problems were unacceptable as the curves could not be used to calculate the concentration of the antigens present in the samples. My hypothesis is that the concentrations of fimbria being examined both in the positive control and the bacterial cultures were too low to be reliably detected when using the reagents that were available. A prudent course of action would have been to increase the concentration of fimbriae in the positive control standard to a level around 10 mg/ml and to likewise concentrate the bacterial cultures to be tested by a factor of 5 or 10. This would have likely brought much stronger reactions that could be analyzed more easily and reliably. However the fluorescency assay, under these conditions, should have reduced the detection limits of the assay. Further research is therefore required to develop a suitable ELIFA.

The next method that was tried was the competitive assay using the affinity purified chicken antibodies. This method worked well in the preliminary trials using the 2 mg/ml standard showing curves that were smooth with low variation. Not surprising was that the response of the bacterial cultures in the assays was weaker (reactivity in the competitive assay is inversely proportionate to the concentration of fimbria present) than the reactivity of the positive control standard (see fig 5.3). The difference was so large that the curves were difficult to compare, because the fluorescency of the standard curve was of far less intense than that of the bacterial sample. Correcting this would be either a matter of concentrating the bacterial suspensions to bring the curves generated by the bacteria and the positive control closer together or decreasing the amount of antigen included in the positive control.

It was hoped that the ELIFA could be optimized to detect extremely low concentrations of target bacteria and so all efforts were aimed at optimizing the assay to detect target bacteria as they would be presented in a typical bacterial culture. Simply decreasing the concentration of the fimbria would have undoubtedly brought the standard curve closer to the range of the bacterial sample curve but to decrease the concentration of the antigens much below 2 mg/ml may have lead to a situation where binding saturation was possible. Binding saturation occurs when the soluble antibodies are present in concentrations sufficient to bind both the soluble and fixed antigens simultaneously. In this case the curve generated by the serial dilution of the soluble antigens would be flat. Correcting the problem of binding saturation by decreasing the concentration of the antibodies could have decreased the sensitivity of the assay and again the curve would likely appear flat. The assay did prove successful using the affinity-purified antibodies; however, there was no supply of affinity purified chicken anti-fimbrial antibodies for strains other than K88 and so another source of antibodies had to be found. Producing more affinity purified antibodies like the ones that had proven successful in this assay thus far was not possible as it would have taken six months to produce them. In that light an attempt was made to use chloroform separated antibodies as a substitute for those purified by an affinity colμMn. The attempt failed. The failure of the chloroform-separated antibodies could perhaps be attributed to the polyclonal nature of the antibodies themselves, as they were generated in chickens with a subunit vaccine which would have yielded some measure of specificity but not enough to serve as a diagnostic tool in this type of assay. They may have lacked the specificity of the purified antibodies that were produced using an elution colμMn that would have concentrated highly reactive antibodies as they were passed through the colμMn. Increasing the concentration of the chloroform-separated antibodies would have run the risk of binding saturation.

Decreasing the concentration of the antibodies may have only served to weaken the reactivity seen and compromise the powers of detection for this assay. Even with the affinity purified antibodies the bacterial culture assays appeared to be at the very limit of detection, bordering on binding saturation by the antibodies. If the standard was decreased in concentration to a similar point it may have become unreliable (exhibiting high variability). It was because of these troubles with optimization that this assay was not continued. The difficulties in optimization would seem to suggest that as a routine diagnostic this assay as conducted would not be very robust or reliable.

5.12 Conclusions on ELIFA/ELISA:

With the exception of the antibody ELISA that had been proven beforehand by previous researchers, all the assays designed and implemented here were found to be unsatisfactory requiring further research and optimization to make them useful in diagnostic applications. Although they did show some early stage successes they were ultimately unsuitable for use in this thesis. Never-the-less it was theoretically possible to apply these assays for their designated tasks and they would have been successfully optimized given enough time, materials and personnel to fully explore their capabilities and shortcomings. The sandwich ELISA may have proven successful if the concentrations of target bacteria were higher. The competitive assay may have proven successful if more affinity purified antibodies were available. Time and again it appears that the failings of these assays were the detection limits of the assays themselves that required higher concentrations of fimbria than were being targeted. Furthermore, given the labor intensive sample preparation and time required for sample testing it was unlikely that the antigen immunoassays as used in this thesis would have ever been incorporated into routine testing techniques. Commercial dip-stick type assays for detection of other microorganisms have been developed that are rapid, sensitive and simple (www.neogen.com). Therefore, it is conceivable that similar assays can be developed to detect *E. coli* pathogens in animal samples. The cross-reference that would have been supplied may not be necessary, given that the correlation between the ELISA and PCR detection of target fimbria and fimbrial genes is near 97% (Ojeniyi, 1994). So given the problems with optimization and the doubtful usefulness of assaying for fimbria directly, the immunoassays for fimbria were dropped all together. Since the survey to characterize and

map the occurrence of ETEC bacteria in Manitoban hog barns was not initiated the serμM assay, although previously proven, was not employed in this study on a large scale.

General Summary and Conclusions

Current trends in livestock production are leading the industry towards the use of effective and sustainable substitutes to antibiotics in order to control enteric pathogens in pigs as well as other animals. These trends are as a result of concerns expressed by the public regarding the emergence of antibiotic resistance in livestock treated with subtherapeutic doses of antimicrobial agents. In order to alleviate these concerns a number of alternative measures of control have been investigated with varying degrees of success. Direct manipulation of the microbial population of the gut has been popular in the past. This manipulation has been achieved by the use of a number of products such as prebiotics that provide selective food sources for non-pathogenic normal flora and probiotics that contain supplemental doses of different species of non-pathogenic normal flora. Other control methods have also been explored including the vaccination of pregnant females or even the progeny directly in order to increase the active immunity of the animals themselves. Though many of these types of products have shown promise, they have yet to yield consistent and effective results to merit their use as stand alone methods for the control of enteric pathogens. However, therapeutic egg antibodies are one product that have demonstrated a definitely beneficial impact on pig production when applied to feed for therapeutic and subtherapeutic treatment of enteric pathogens. These antibodies are highly effective at eliminating the populations of enteric pathogens from the gut by inhibiting their colonization and allowing their passage with normal fecal excretion. They are as specific as they are effective and this is a benefit in that it prevents the broad-spectrum destruction of normal flora along with the pathogens. That specificity also means that the antibodies need to be accurately applied and to do so requires a diagnostic system to verify the presence of

target organisms in the animal in question. The goal of this thesis was to provide such a diagnostic system using genetic analysis with the polymerase chain reaction and a molecular detection system using the ELISA immunoassay system.

The PCR system was used with good success in detecting the presence of target pathogen in animals specifically inoculated with a predetermined pathogen. It was also able to detect target pathogens in animals naturally infected but in this case the rate of false negative results was more difficult to determine. Although the assay is effective in detecting the presence of target pathogens it does require optimization. The application of high quality reagents supplying superior reaction conditions is vital. Perhaps more important, however, is the optimization of primer ratios in multiplex assays testing for multiple genes of interest simultaneously. If there is an imbalance in the primer concentration, activity, or both, the sensitivity and accuracy of both primers can be compromised and the method invalidated. It was for this reason that the interactions of the primers needed to be carefully optimized to avoid masking of one primer's activity by another. Once optimized, however, the multiplex PCR system was efficient and accurate for the simultaneous detection of multiple virulence factor genes.

A non-competitive ELISA immunoassay system was applied and optimized for the detection of target anti-ETEC antibodies. It appears to be a robust and useful technique for establishing a history of infectious disease exposure in an animal by quantitating the activity of antibodies specific for targeted ETEC surface antigens in the serum of pigs. The presence of such antibodies would indicate that an animal in question had encountered the pathogen(s) in the past, was infected and developed an effective immunity against the

pathogen. A situation may arise where a production facility is having troubles with scouring and the PCR is unable to detect a potential agent responsible at the time animals are being tested. This failure of the PCR could be attributed to the use of antibiotics killing off the pathogens or the spontaneous resolution of transient scouring, in both cases the levels of ETEC bacteria in the fecal swabs may be below the level of detection for the PCR protocol used. In this case the antibody profile of animals exposed to the pathogen but not currently exhibiting clinical scours would remain unchanged. The ability to detect past ETEC infections adds a valuable dimension to the PCR assay system that can only determine the nature of pathogens if they are present in detectable levels in the fecal swabs of animals. The antibody ELISA does not require the presence of viable pathogens at the time of testing and the activity of antibodies present could give clues as to the severity and or time of infection. Logically speaking the higher the activity of antibodies present the more severe and/or the more recent the period of infection.

The ELISA immuno assay system was also examined for its ability to directly detect surface proteins on specific *Escherichia coli* pathogens. However, the success of these assays was limited. The assays that were successful were conducted on a preliminary basis on purified and concentrated surface antigen isolates. The ELISA detection systems as used in this study were not able to detect target pathogens at the concentration that would be present in a normal bacterial culture. In order to increase the repeatability, reliability and accuracy of the antigen ELISA's the bacterial cultures examined, as tested in this thesis, would need to be purified and concentrated to provide the numbers of target organisms required for positive identification. Since there should be a high correlation between the presence of virulence factor genes and their expression in cell culture it seems pointless and

time consuming to pursue the development of both types of assays for one detection system. That is not to say that ELISA assays are incapable of pathogen detection, they certainly are but the fact remains that the preparation and application time required if far greater than that seen with the PCR and with no apparent added benefit. For simplicity's sake the PCR can stand-alone as a detection method for pathogenic *E. coli* once the assay has been optimized and verified for accuracy.

Future Research

There were a number of problems encountered with optimizing the ELISA system to detect the presence of target ETEC surface antigens, the assays that were successful on a preliminary basis were not very robust and difficult to reproduce. The problem with this was the fundamental approach that was designed to directly detect the presence of the target antigen. Another approach would be to test for the *absence* of target antigen in a normal noncompetitive ELISA. The protocol would be identical to a standard ELISA for the activity of antibodies except that with the exception of the negative control the antibodies would be mixed with a sample of suspected ETEC antigens. The irreversible binding that would occur between the antibodies and the complementary antigen would serve to decrease the number of available antigen binding sights in a given antibody solution. When an antibody ELISA is performed on that antigen exposed sample the binding of complementary antigens would become apparent in terms of a decrease in activity of the antibody solution. The level of decrease would then be quantitated based on comparison to the negative control with an arbitrary activity of 1, antigen exposed samples would have an activity expressed as a percentage of 1. Samples with an activity of 1 contain no complementary antigen; samples with an activity of 0.99 to 0 have complementary antigen. Since the presence of target antigen decreases the antibody activity, the lower the activity of the sample the higher the concentration of the antigen present. A system like this should be robust and far easier to use than the sandwich or conventional competitive ELISA's. In order to increase the sensitivity of the assay the bacterial samples could be prepared using a simple pH extraction of fimbriae. This would serve to decrease the interference from the

whole bacteria, cellular debris from cell lysis and bacterial metabolites while concentrating the fimbria targeted.

The direct PCR assays were not as reliable as they could be, to increase the sensitivity and reliability of those assays some means of DNA extraction needs to be applied to resolve the false negative results encountered. In terms of the enriched assays there were some potentially false negative results encountered when testing with the PCR. One way of potentially reducing these is by sorting the target bacteria present from the non-target bacteria using a strain specific harvesting device. One such device would employ PVC strips that have been coated with antibodies specific for the target ETEC fimbriae under conditions as sterile as possible. When these sterile antibody coated strips are added to a mixed culture they should be able to differentially bind to target ETEC bacteria based on the antibody specificity. After a period of sufficient for growth and binding of the bacteria has elapsed the antibody strips could be transferred to fresh sterile media. Theoretically speaking, the only bacteria that should be transferred to the fresh media (after the strips have been washed in sterile buffer) are those that are complementary for the antibodies attached to the strips. The bacteria adhering to the strips are not killed by the antibodies and could be re-enriched in the fresh media as a semi pure culture. The PCR may be able to detect the presence of target pathogens more easily in a culture of this type compared to a raw mixed culture inoculated by straight fecal swabs.

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