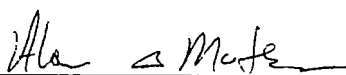


To the Graduate Council

I am submitting herewith a thesis written by Mark Ashburn Franklin entitled "A PCR-Based Method of Detection and Differentiation of K88 *Escherichia coli*" I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Animal Science


Alan Mathew, Major Professor

We have read this thesis
and recommend its acceptance.


Frank B. Masincup

Accepted for the Council


Associate Vice Chancellor and
Dean of The Graduate School

A PCR-BASED METHOD OF DETECTION AND
DIFFERENTIATION OF K88 *ESCHERICHIA COLI*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Mark Ashburn Franklin

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ABSTRACT

The objective of this study was to develop a Polymerase Chain Reaction (PCR)-based method to detect and differentiate between *Escherichia coli* possessing genes for the expression of three antigenic variants of the fimbrial adhesin K88 (K88ab, K88ac, and K88ad). Five primers were designed that allowed detection of K88 *E. coli*, regardless of antigenic variant, as well as the separate detection of ab, ac, and ad variants. Primers AM005 and AM006 were 21 base-pair oligomers that corresponded to a region of the K88 operon that is common to all 3 antigenic variants. Primers MF007, MF008, and MF009 were 24 base-pair oligomers that matched variable regions specific to ab, ac, and ad variants, respectively. Using primers AM005 and AM006, a PCR product was obtained that corresponded to a 764 base-pair region within the large structural subunit of the K88 operon common to all 3 antigenic variants. Primer AM005 used with either MF007, MF008, or MF009 produced PCR products approximately 500 base-pairs in length from within the large structural subunit of the K88 operon of the 3 respective antigenic variants. Fragments were identified by rates of migration on a 1% agarose gel relative to each

other as well as to fragments derived from BstEII-digested lambda DNA. This PCR-based method compared well with ELISA and Western Blot tests for the ability to differentiate between the antigenic variants. K88 *E. coli* were differentiated from among laboratory strains and detected in ileal samples taken from cannulated pigs challenged with a known K88 variant. K88 *E. coli* were also detected from fecal swabs taken from newly weaned pigs, thus confirming that this PCR-based test could provide a convenient clinical assay for the detection of K88 *E. coli*. Detection and differentiation of K88 *E. coli* using general and specific primers were successful. PCR methods of detection should permit identification of K88 antigenic variants regardless of the level of expression of the antigen.

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1. INTRODUCTION

K88 *E. coli* have been implicated as diarrheal agents in newly weaned pigs (Smith and Jones, 1963). These bacteria are known to colonize the small intestine in large numbers and can have a detrimental effect due primarily to the concurrent release of St and Lt toxins (Smith and Gyles, 1970). These toxins cause water and electrolytes to be secreted from the intestinal tissues into the lumen. The ability to colonize the small intestine has been attributed to fimbrial adhesins or pili, the genes of which are known to be carried on a plasmid (Orskov and Orskov, 1966). Differentiation between *E. coli* and other sources of diarrhea is essential for proper detection and control of the disease. Simple seroagglutination tests (SAT) have been used in the past to identify K88 *E. coli*; however, such tests require sufficient antigen production for success and thus are not always reliable in identifying these pathogens (Mullaney et al., 1991). Other methods such as ELISA and indirect fluorescent antibody tests (IFAT) have also been used. These tests allow better detection but still require large amounts of antigen production and/or sacrifice of the animal (Mullaney et al., 1991). Thus, the objective of this

study was to develop a PCR technique that was more reliable and simpler to perform for the diagnosis of K88 *E. coli*.

2. LITERATURE REVIEW

Prevalence of Pathogenic *Escherichia coli*

Escherichia coli (*E. coli*) have been recognized as a pathogen in man and animals for a number of years (Hentges, 1970; Mills et al., 1982; Rose et al., 1987; Casey et al., 1990). First described by Theodor Escherich in the late 1800's (Bertschinger et al., 1992), these gram negative bacteria are now known to be responsible for diarrhea in both man and swine (DeGraaf, 1990; Bertschinger et al., 1992). In humans, diarrhea affects primarily young infants and children (Sherman et al. 1989). In swine, diarrhea also primarily affects the young, having its greatest effect on newborn and weanling pigs (Moseley et al., 1986). *E. coli* were determined to be the etiologic agent in 48% of swine diarrhea cases in the United States (Bergeland, 1980). Bacterial counts have shown that pathogenic strains of *E. coli* greatly outnumber non-pathogenic strains in diseased versus healthy pigs (Smith and Jones, 1963). K88 *E. coli*, a pathogenic strain possessing specific adhesive antigens, have been implicated as a major source of diarrhea in postweaned pigs, with one study showing 72% of strains

isolated from pigs greater than 24 days old being K88 positive (Wilson and Francis, 1986).

Economic Impact of Pathogenic *E. coli*

Postweaning diarrhea (PWD) is a major source of economic loss for swine producers with scours accounting for the majority of mortality and morbidity following weaning (USDA, 1992). Based on a national annual production of 98 million pigs, nursery losses are estimated to account for over 2.3 million pig deaths per year (USDA, 1992). If one conservatively assumes a \$20 production cost per weaned pig and a potential net profit of \$10 per finished pig (Rawls, 1993), the monetary loss to swine producers exceeds 70 million dollars annually. However, this is not the only source of economic loss. Poor feed efficiency, stunted growth, and chronic diarrhea result in higher feed, fuel, and medication costs, increased days to market, and an interruption in the flow of production.

Control of Pathogenic *E. coli*

Antibiotics have been used in animal feeds for the past 40 years (Visek, 1978). These compounds have been shown to

have growth promotant benefits which are thought to be due to the effects on intestinal microflora (Coates et al., 1963). However, antibiotic use also has potential problems due to resistance which can develop from long term therapeutic and subtherapeutic use (Dawson et al., 1984; Langlois et al., 1984). Another method for control of bacterial diseases in swine includes all-in all-out production schedules which allow for cleaning and sterilization of facilities. These schedules are most common in farrowing and nursery units where the young pig experiences its first exposure to bacterial populations. Vaccination of sows is also a very common method to control *E. coli*. However, problems also exist due to bacterial adaptations to these vaccines. Guinee and Janssen (1979) stated that the K88ad *E. coli* variant may have arisen from selective pressure due to the use of K88 *E. coli* combatant vaccines.

Types of Pathogenic *E. coli*

In the presence of appropriate predisposing conditions, pathogenic *E. coli* proliferate and cause diarrhea by means of specific virulence factors (Fairbrother, 1992). Several

types of pathogenic porcine *E. coli* exist, including: enterotoxigenic *E. coli* (ETEC), verocytotoxigenic *E. coli* (VTEC), and attaching and effacing *E. coli* (AEEC) (Fairbrother, 1992). *E. coli* isolated from humans have also been described as enteropathogenic *E. coli* (EPEC) (Sherman et al. 1989), although humans may be affected by other types as well. AEEC attach to the intestinal mucosa and cause lesions similar to those observed for EPEC (Fairbrother, 1992); however, unlike other diarrheagenic *E. coli*, EPEC do not elaborate heat-labile and heat-stable enterotoxins (Sherman et al. 1989). In contrast to EPEC, ETEC produce heat-labile and heat-stable toxins as mechanisms of virulence (Moon et al., 1986; Broes et al., 1988). VTEC also produce toxic compounds known as shiga-like toxins, due to their similarity to the toxin produced by *Shigella dysenteriae* (Bertschinger and Nielsen, 1992). Although mechanisms and virulence factors differ, interaction with mucosal surfaces is a common initial step in infection (Casey et al., 1990).

ETEC Infections

Diarrhea caused by enterotoxigenic *E. coli* (ETEC) is associated with several pilus antigens which include K88 (F4), K99 (F5), 987P (F6), and F41 in neonatal swine (Nagy et al., 1992). These pili appear to be restricted to a limited number of serogroups including O8, O147, O149, and O157 (Wilson and Francis, 1986; Harel et al., 1991). K88 *E. coli* are known to produce proteinaceous filaments, or pili, which allow their attachment to the small intestinal mucosa (Smith and Linggood, 1971; Jones and Rutter, 1972; Nagy et al., 1977; Moon et al., 1979). These pili are thought to be vital for colonization of the small intestine because they promote adherence that resists clearing actions of the intestinal tract (Isaacson and Richter, 1981; Gaastra and DeGraaf, 1982; Isaacson, 1985; Sarmiento et al., 1988). These clearing actions are the result of peristaltic movements of the small intestine and have their greatest force in the anterior portion of the intestinal tract (Jann and Hoschutzky, 1990). Adhesive pili are thought to consist of proteinaceous adhesive subunits which have their receptor sites blocked by their organization into fimbria. Only the most terminal portions are exposed, providing a site for

adhesion to occur (Jann and Hoschutzky, 1990). Receptors for fimbria are found on the brush border mucus membranes lining the intestine (Sellwood et al., 1975). Recently two glycoproteins have been identified in the porcine brush border that bind with the K88ac variant, possibly comprising part or all of the receptor binding complex. These receptor glycoproteins have been shown to be 210 and 240 kDa in size, and bind to both K88ac *E. coli* and purified K88ac adhesin (Erickson et al., 1992). However, not all pigs possess adhesin receptors, as their presence is conferred by an autosomal dominant gene (Gibbons et al., 1977). The homozygous recessive genotype is resistant to K88 infection but is susceptible to other pilus antigen types (Smith and Huggins, 1978). *E. coli* possessing other pilus antigens have been shown to cause diarrhea in neonatal pigs as well, but neither K99 nor 987P have been found on enteropathogens from pigs greater than 2 weeks of age (Moon et al., 1980).

Colonization of *E. coli*

The intestinal tract of newborn piglets is sterile at birth (Kenworthy and Crabb, 1963; Katouli et al., 1995). The tract quickly becomes colonized shortly after birth from

the environment surrounding the pig (Smith and Jones, 1963; Kenworthy and Crabb, 1963). Colonization is thought to be enhanced by the high pH of the intestinal contents throughout the tract in the neonatal pig (Smith and Jones, 1963; Smith, 1965). In the study by Smith and Jones (1963), pH was determined in the stomach of two pigs at 6 and 55 hours postpartum. The stomach pH of these pigs was 5.3 and 3.9, respectively. They also found that after weaning an increase in intestinal pH occurs, which possibly allows for recolonization of bacteria that initially colonized the newborn intestinal tract. In more recent studies using 21 day old weaned pigs, Mathew et al. (1994) has shown an increase in ileal pH by 3 days postweaning. However, the increased postweaning ileal pH returned to near preweaning levels by 35 days of age. These investigators postulated that the increase in pH could be due to the common initial fasting period observed with many newly weaned pigs (Mathew et al., 1994). Kenworthy and Crabb (1963) stated that even in some eight week old pigs, the complement of digestive enzymes may not be sufficient to deal with radical changes in diet. Thus, these bacteria could utilize the substrate more efficiently for the short

period of time immediately postweaning, allowing them to obtain high population densities, especially as weaning ages are decreased. After pH begins to decline, a decrease in the numbers of bacteria occurs, except for lactobacilli which thrive in acidic conditions (Smith and Jones, 1963). However, studies have shown the presence of *E. coli* in the intestinal tract in the absence of a disease state (Kenworthy and Crabb, 1963; Hinton et al., 1985). Smith and Jones (1963) also noted no differences between the pH of the chyme in the diseased piglets and that of their apparently normal littermates. Thus, colonization by pathogenic *E. coli* appears not to be totally dependent on pH, but also on the initial presence of pathogenic *E. coli* in the alimentary tract. The above study also demonstrated during disease outbreaks, pathogenic *E. coli* proliferated with little disturbance of other bacteria normally present. It has also been shown that animals with a greater diversity of microflora are less susceptible to disease resulting from bacterial infections. Kuehn et al. (1993) demonstrated a decrease in coliform diversity during and after a diarrheal outbreak, and piglets that died carried bacterial populations with lower diversity than those which survived

the outbreak. This diversity may involve several species including but not limited to *E. coli*, *Clostridium welchii*, streptococci, yeasts, and lactobacilli (Smith and Jones, 1963). Colonization of ETEC has been shown to occur progressively from the posterior portion of the small intestine to the anterior end (Sarmiento *et al.*, 1988), rather than from the stomach toward the posterior portion of the small intestine. In conclusion, colonization is enhanced by adhesive pili that give ETEC several advantages including resistance to the rinsing and cleansing action of body fluids, evasion of host defenses, and improved acquisition of nutrients from their environment (Jann and Hoschutzky, 1990).

Adhesive Factors

At least three distinct antigenic variants of K88 *E. coli* have been identified and are termed K88ab, K88ac, and K88ad (Guinee and Janssen, 1979). It has been postulated that the K88ad variant developed due to immunologic pressure upon K88 bacterial populations as a result of large-scale vaccinations with anti-K88 vaccines (Guinee and Janssen,

1979). However, this theory has been refuted in the literature (Moon, 1990). K88 antigens consist of a constant determinant designated as a, and variable determinants designated as b, c, and d (Guinee and Janssen, 1979). It has been suggested that K88ac is the predominant K88 variant in the major pork-producing regions of the United States (Westerman et al., 1988). The structural gene for the K88 antigen is located on a transmissible plasmid (Orskov and Orskov, 1966), which is commonly associated with at least two other virulence plasmids designated ENT (enterotoxin) and HLY (hemolysin) (Smith and Linggood, 1971). It has been shown that induction of both the K88 antigen and enterotoxin plasmids into an *E. coli* strain from the normal flora of a pig converts the recipient strain to pathogenicity, but neither plasmid alone will do so (Smith and Linggood, 1971). It has also been shown that the ability to utilize raffinose is transferred in high correlation with the K88 antigen (Smith and Parsell, 1975). However, this appears to be of no functional significance to the virulence of K88 *E. coli*, although it may be of significance in selecting for K88 bacteria when grown on substrate containing raffinose. This correlation of transfer was later confirmed by other workers

and these properties were shown to reside on a single plasmid (Shipley et al., 1978). Several investigators have shown that adhesive pili are not produced as abundantly in vitro (Nagy et al., 1977; Moon et al., 1980; Francis, 1983). This may be due to differences in growing conditions in vitro versus in vivo (DeGraaf, 1988). Nagy et al. (1977) also suggested that loss of in vitro adhesiveness occurred during a 2 month laboratory storage period.

Mechanisms of Toxin Production

EPEC secrete toxins which stimulate the release of water and electrolytes into the lumen of the small intestine (Moon et al., 1986; Broes et al., 1988). These toxins include the thermostable Sta and Stb enterotoxins and the thermolabile LT enterotoxin (Gaastra and DeGraaf, 1982). The mechanisms of these toxins are thought to differ, with the Sta toxin being reported to involve the activation of intestinal guanylate cyclase (Dreyfus et al., 1984). The LT toxin apparently has its effect through stimulation of adenylate cyclase in intestinal and nonintestinal cells (Dreyfus et al., 1984). The mechanism of action of Stb is still unknown; however, it is thought to stimulate cyclic

nucleotide-independent fluid secretion in the gut (Kennedy et al., 1984).

Methods of Detection

Detection of K88 *E. coli* from an infected animal is an essential step in recognizing the disease. Several tests have been employed for detection of K88 *E. coli*. These tests include: seroagglutination tests (SAT) (Francis, 1983), enzyme-linked immunosorbent assay (ELISA) (Francis, 1983), indirect fluorescent antibody tests (IFAT) (Francis, 1983), immunomagnetic particles (IMP) (Lund et al., 1988), DNA probes for fimbrial and enterotoxin genes (DNA) (Harel et al., 1991), and a polymerase chain reaction (PCR) test for the heat-stable enterotoxin gene (Hornes et al., 1991). The SAT relies on visible agglutination of the reaction mixture (Mullaney et al., 1991), making this test subject to relative comparison by the observer for analysis. Inconsistent results with the SAT have suggested that this test was unreliable for the detection of K88 pilus antigens (Mullaney et al., 1991). The ELISA requires the purification of pili as well as the production of antiserum (Mills et al., 1982). Purification of pili requires that

E. coli be grown in large quantities *in vitro* which may cause decreased pili production due to the artificial conditions of growth (Nagy et al., 1977), which do not truly mimick the environment in the piglet small intestine. Production and isolation of antiserum can be a lengthy procedure, requiring up to 1 month before harvesting from the host animal (Mills et al., 1982). The IFAT is performed on ileal impression smears and requires a section of excised intestine; however, this test does not require bacterial culture as is needed for the two previously described tests (Francis, 1983). The IMP procedure is a more rapid analysis using antibody-coated magnetic beads as a means of separation; thus still requiring antibody specificity. Proper washing of the coated beads, and sufficient pili density are also important requirements (Lund et al., 1988). DNA probes appear to be much more specific than the above mentioned tests but require the handling of radioactive material to label the probes (Harel et al., 1991). PCR analysis does not require the use of radioactivity, is very specific, and does not require antibody production. However, care must be taken to avoid false positive results

and the presence of substances such as organic matter that might interfere with DNA replication (Hornes et al., 1991).

3. MATERIALS AND METHODS

Strains: Strains used in this study included isolates from the three known antigenic variants as well as a K99 strain for a negative control (Table 1).

Primers: Primers were designed to hybridize with a region of the K88 operon that codes for the large structural subunit of the K88 fimbrial adhesin (Figure 1). Primer AM005 (sense) was a 21-bp oligomer: 5'-GGT GAT TTC AAT GGT TCG GTC-3', corresponding to an upstream region common to all three variants. Primer AM006 (antisense) was a 21-bp oligomer: 5'-ATT GCT ACG TTC AGC GGA GCG-3', corresponding to a downstream region common to all three variants. Primer MF007 (antisense) was a 24-bp oligomer: 5'-TGC AGC ACC CGA AAC AGT CGT CGT-3', corresponding to a downstream region specific to the ab variant. Primer MF008 (antisense) was a 24-bp oligomer: 5'-CCC AGC CGA CGA TTC AGA ACC CCT-3', corresponding to a downstream region specific to the ac variant. Primer MF009 (antisense) was a 24-bp oligomer: 5'-TGC AGA ATT CTG AAC ATT CGT CGG-3', corresponding to a downstream region specific to the ad variant. MF primers hybridized to a region upstream of the AM006 site and were

Table 1. Antigenic makeup and source of tested strains

Designation	Antigenic variant
K12:4456*	K88ad
K12*	K88ac
K12*	K88ab
O8:3030-2*	K88ac
O8:263*	K88ab
O8*	K88ad
2037#	K88ad
2033#	K88ab
2034#	K88ab
2029#	K88ab
2031#	K88ad
O157:H13 ⁺	K88ac
O101:NM ⁺	K99
K12/pMK 005\$	K88ac

* Kindly provided by David Francis, Dept. of Vet. Sci., South Dakota State Univ., P.O. Box 2175, Brookings, SD 57007-1396.

Kindly provided by Evelyn Dean-Nystrom, USDA, NADC, P.O. Box 70, Ames, IA 50010.

+ Kindly provided by The *E. coli* Reference Center, The Pennsylvania State Univ., 105 Henning Building, University Park, PA, 16802.

\$ Kindly provided by Stephen Moseley, Dept. of Microbiology, Washington State Univ., Sc.42 F324, Seattle, WA 98195.

	Primer AM005														
	1													15	
K88ab	TGG	ATG	ACT	GGT	GAT	TTC	AAT	GGT	TCG	GTC	GAT	ATC	GGT	GGT	AGT ---
K88ac	TGG	ATG	ACT	GGT	GAT	TTC	AAT	GGT	TCG	GTC	GAT	ATC	GGT	GGT	AGT ---
K88ad	TGG	ATG	ACT	GGT	GAT	TTC	AAT	GGT	TCG	GTC	GAT	ATC	GGT	GGT	AGT ---
K88ab	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ac	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ad	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ab	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ac	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ad	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	160	Primer MF007												175	
K88ab	GGT	GGT	TTG	ACG	ACG	ACT	GTT	TCG	GGT	GCT	GCA	CTC	ACG	AGT	GGG ---
		Primer MF008													
K88ac	GGT	GGT	TTG	CCG	AGG				GGT	TCT	GAA	TCG	TCG	GCT	GGG ---
		Primer MF009													
K88ad	GGT	GGT	TTG	CCG	ACG	AAT	GTT	CAG	AAT	TCT	GCA	CTC	CCG	GGT	GGG ---
K88ab	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ac	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
K88ad	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	250	Primer AM006												265	
K88ab	AGC	ACT	CAG	TGG	AGC	GCT	CCG	CTG	AAC	GTA	GCA	ATA	ACT	TAT	TAC ---
K88ac	AGC	ACT	CAG	TGG	AGC	GCT	CCG	CTG	AAC	GTA	GCA	ATA	ACT	TAT	TAC ---
K88ad	AGC	ACT	CAG	TGG	AGC	GCT	CCG	CTG	AAC	GTA	GCA	ATA	ACT	TAT	TAC ---

Figure 1. Schematic representation of the primer positions on the K88 genome, showing the regions of difference between the 3 variants.

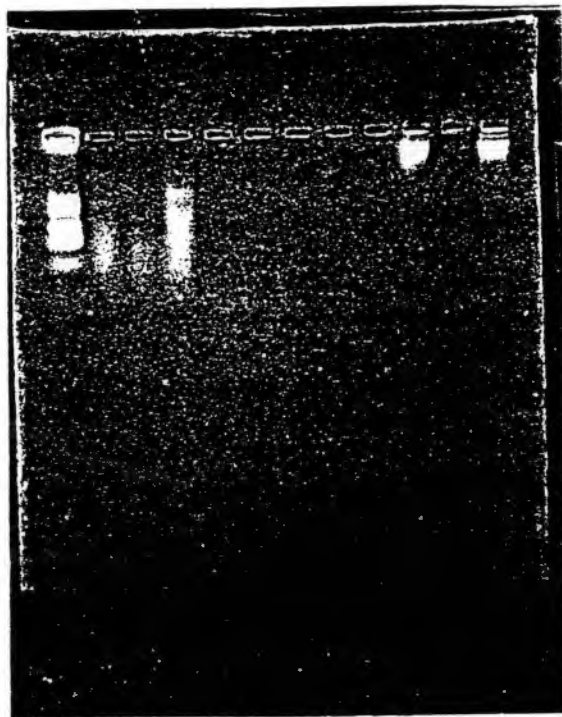
selected based upon regions of difference between the 3 antigenic variants (Josephsen et al., 1984).

Elucidation of thermal cycling conditions: The thermal cycler used in this study required optimization to perform adequate cycling times. Due to differences between the block temperature and the probe temperature, allowances were made to achieve the necessary cycling times. The integral timer began countdown when the preset block temperature was reached, not when the probe temperature was reached thus, additional time had to be added to the set time to achieve the necessary times at the correct temperatures. To achieve this, several trial runs had to be conducted while monitoring the actual temperature of the probe.

PCR Conditions: Reactions were performed using a standardized buffer (Invitrogen, San Diego, CA) containing 300mM Tris, pH 8.5, 75mM $(\text{NH}_4)_2\text{SO}_4$, and 7.5mM MgCl_2 . Each deoxynucleoside triphosphate (Boehringer-Mannheim, Germany) was included at 2.5 mM, along with 1 mM of each primer (Oligos, Wilsonville, OR), 1.15ng of plasmid DNA, and 1 unit of Taq DNA polymerase (Promega, Madison, WI) in a final reaction volume of 50ul. Reactions were overlaid with 50ul of mineral oil to prevent evaporation. Thermal cycling

consisted of an initial denaturation step of 3.5 min at 94⁰C, followed by 35 cycles of 1.5 min at 94⁰C, 2.5 min at 55⁰C, 4 min at 72⁰C, and a final elongation period of 7 min at 72⁰C in a GTC-2 Precision Scientific thermocycler (Precision Scientific, Chicago, IL).

Plasmid DNA Extraction: At the initiation of this study, plasmid DNA was extracted from *E. coli* cultures using a Wizard Maxiprep kit (Promega, Madison, WI). This procedure was found to be unsatisfactory because of the degradation of the extraction products even when stored at - 80° C (Figure 2). A phenol-chloroform extraction was then performed but was found to be too time consuming and the quantity of the remaining product was not sufficient for repeated trials. It was found that plasmid DNA extraction from individual colonies could be achieved more efficiently using an Instagene Matrix (Bio-Rad, Hercules, CA). Plasmid product degradation was still a problem, but the time required for the procedure was reduced and the amount of product was sufficiently greater, thus making this the optimal choice for plasmid DNA extraction. This process consisted of an initial incubation for 30 min with 200ul of the Instagene Matrix. After vortexing, the mixture was



This picture shows the degradation that takes place after one week of storage at -80 degrees Celsius.

Figure 2. Photograph of plasmid DNA after isolation with Wizard DNA Maxiprep.

boiled for 8 min in a waterbath. Five microliters of this mixture was used in each PCR reaction. Fresh colony preps were prepared before each PCR reaction to ensure that the plasmid DNA had not degraded. Colonies were chosen randomly from the respective plates and positive colonies were stored at -80°C for later analyses. Isolates from live were grown on lactose MacConkey media (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) and 30 colonies were transferred to 5% sheep's blood agar plates (BBL, Cockeysville, MD) to detect hemolytic activity. Three colonies were selected from the blood agar plates with preference given to hemolytic colonies when present. Laboratory strains were prepared as discussed above.

Challenge of Live Pigs: Cannulated pigs from a separate challenge study were used to isolate K88 *E. coli*. This method for ileal cannulation has been discussed previously in the literature (Mathew et al., 1993; Mathew et al., 1994). Briefly, this procedure consisted of surgically implanting a T-type cannula approximately 10 cm proximal to the ileo-cecal junction. This was achieved consistently by locating the first loop proximal to the ileo-cecal junction and using this site as a landmark for

location of the cannula. After recovery, the pigs were placed back on the sow to continue nursing for approximately 5 days to allow for an adjustment period. The 21-day old pigs were then weaned and sampled at appropriate times as determined by the experimental protocol. Pigs were challenged with a wild type strain known to carry the plasmid for adhesion (O157:K88ac). The procedure consisted of growing the isolate overnight in 50 ml of Luria broth. The culture was then spun at 15000 x g for 10 min in a Beckman JH-20 centrifuge (Palo Alto, CA) to pellet the cells. The supernatant was removed and 50 ml of phosphate buffered saline (PBS) was added to the cells. The mixture was vortexed and spun again. To the remaining pellet, 50 ml of PBS was added and, after vortexing, 2 ml of this mixture was orally administered to each pig. Challenge cultures were enumerated on MacConkey media, and each pig was determined to have received a minimum of 10^9 colony-forming units per ml of culture. Ileal digesta, collected from day 1 to day 21 post-challenge, was plated onto MacConkey media and 3 individual colonies were subjected to the PCR-based test. Fecal swabs from those same pigs were also plated

onto MacConkey media and 3 individual colonies were subjected to the PCR test.

Visualization of Products: One microliter of a 20% glycerol loading buffer was combined with 10ul of each PCR product prior to loading on a 1% agarose gel for 15 min at 58 V. The amplified products were visualized by ethidium bromide stain and UV light and their lengths verified by a digested lambda DNA standard run simultaneously on the agarose gel (Figure 3).

Lambda DNA
BstEII (kb)

--- 8.45
--- 7.24
--- 6.37
--- 5.69
--- 4.82
--- 3.68

--- 2.32
--- 1.93

--- 1.37
--- 1.26

--- .70

--- .22

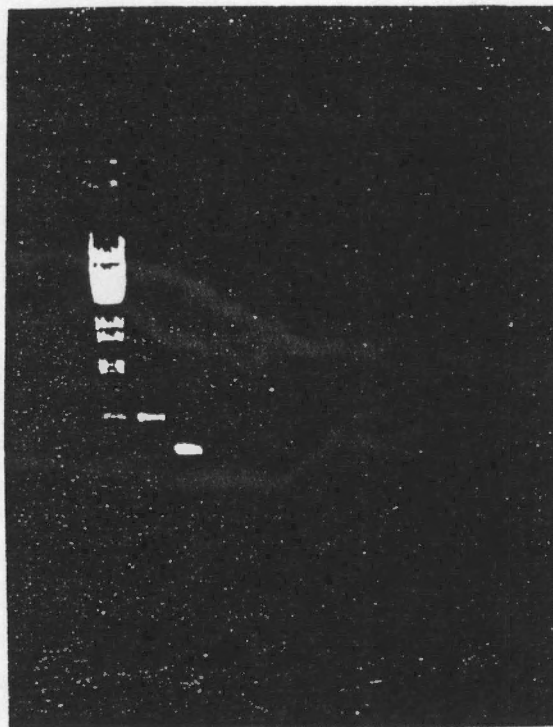
--- .12

Figure 3. Schematic representation of the banding pattern for the DNA standard used in this study to compare with PCR products.

Controls: An O101:K99 *E. coli* (Table 1) was used as a negative control. As further controls, each of the specific primers were run with templates from the non-matched variants. A cloned strain pmK(005) (Kehoe et al., 1981) was used to confirm the specificity of the general primers (AM005 and AM006) for detection of K88 *E. coli*, regardless of antigenic variant. Reactions were also performed omitting the polymerase to ensure that possible excessive template DNA had not been mistakenly identified as a positive amplification. ELISA (Voller et al., 1980) and Western Blot (Burnette, 1981) analyses were used to compare efficacy of the PCR-based test to differentiate between variants.

4. RESULTS

Resultant PCR products corresponded to expected lengths from within the large structural subunit of the K88 operon. Primers AM005 and AM006 produced a product corresponding to a 764-bp region that was common to all 3 antigenic variants. When primer AM005 and one of the MF primers were used, a PCR product of approximately 500-bp was obtained (Figure 4). The lengths of these products were verified by fragments derived from BstEII digested lambda DNA included as a standard in each gel. None of the reactions produced amplification when using the K99 template. Amplification products were also not produced when variants and specific primers were incorrectly matched. The 005 strain showed an amplification product with primers AM005 and AM006. No amplification products were visible in any of the reactions when the polymerase had been omitted. A product was deemed positive when it produced a distinctive band consistent with the expected migration on the agarose gel and as determined by comparison to the appropriate DNA fragment standards. The results of the ELISA, Western Blot, and PCR-based tests are summarized in Table 2. These data show results for 9



From the left of the picture, Lane 1 shows the DNA standard used for comparison. Lane 2 shows an amplification product using primers AM005 and AM006. Lane 3 shows an amplification product using primers AM005 and MF008.

Figure 4. Photograph of PCR products using general and specific primers.

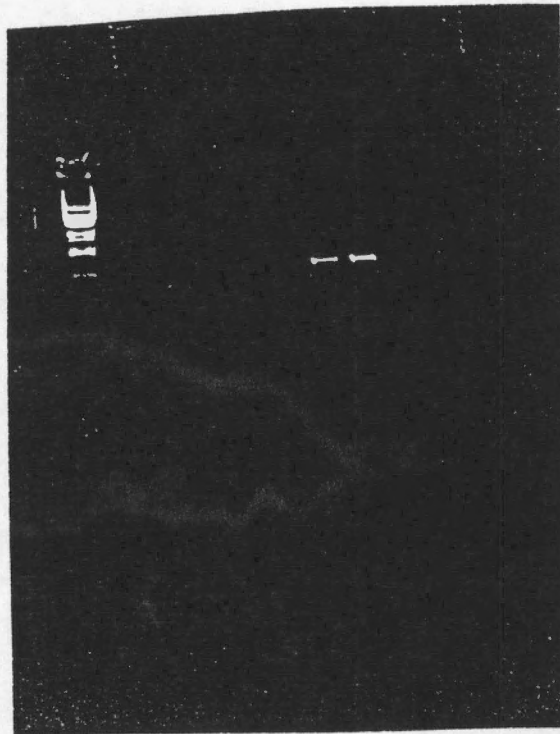
Table 2. ELISA, Western Blot, and PCR results

		ELISA			Western			PCR			
								ab	ac	ad	K88
								Primers	Primers	Primers	Primers
		a	c	d	a	c	d	AM005	AM005	AM005	AM005
Strain	Hemolysis	Mab	Mab	Mab	Mab	Mab	Mab	MF007	MF008	MF009	AM006
08 3030-2	+	+	+	-	+	+	-	-	+	-	+
O157 H13	+	+	-	-	+	-	-	-	+	-	+
K12ac	-	+	-	-	+	-	-	-	+	-	+
2037	+	+	-	+	+	-	+	-	-	+	+
2031	+	+	-	+	+	-	+	-	-	+	+
K12ad	+	+	-	+	+	-	+	-	-	+	+
08 263	+	+	-	-	+	-	-	+	-	-	+
K12ab	-	+	-	-	+	-	-	+	-	-	+
2034	+	+	-	-	+	-	-	+	-	-	+
O101 K99 NM	-	-	-	-	-	-	-	-	-	-	-
005	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+

Mab= Monoclonal antibody

Mab= Monoclonal antibody

variants, as well as the K99 negative control subjected to all 3 tests. The a determinant was detected by all 3 methods for all 9 variants. The 3 ad variants showed 100% agreement between all 3 tests. One of the ac variants showed agreement for all 3 tests. Western and ELISA tests were not available for the b determinant and thus only results from the PCR test are shown, however the ab variants were determined not to be ac or ad variants, based on ELISA and Western Blot analysis. The ab variants were also determined to contain the a determinant by the ELISA and Western Blot techniques. K88 *E. coli* were detected among isolates from live pigs involved in the challenge study as well (Figure 5). Isolates from fecal swabs of those same pigs also tested positive for K88 *E. coli*.



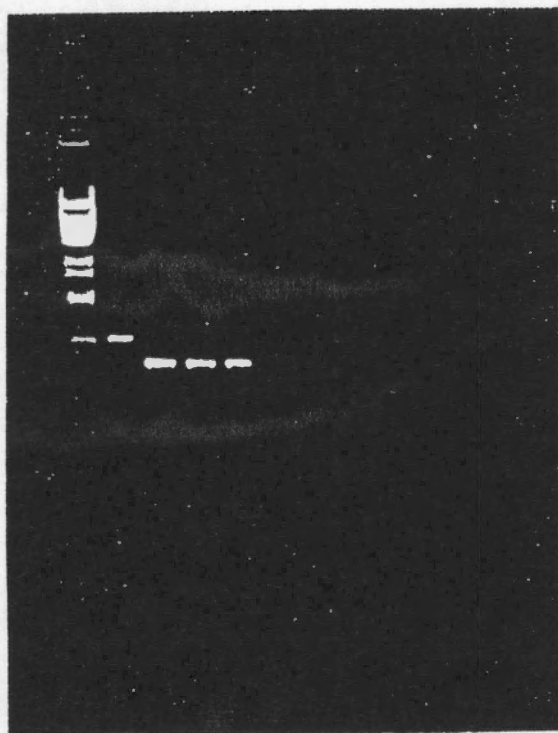
Lane 1 shows the DNA standard used for comparison. Lanes 7 and 8 show amplification products using primers AM005 and AM006.

Figure 5. Photograph of PCR products amplified from *E. coli* colonies taken directly from pig fecal swabs using general primers to identify *E. coli* isolates.

5. DISCUSSION

Our PCR-based technique is not dependent on the level of expression of the antigen. Thus, detection of ETEC that carry the adhesin plasmid but may not be expressing sufficient quantities of fimbria should be improved over ELISA and Western Blot techniques. We were able to detect K88 *E. coli* in challenged pigs that did not appear to have symptoms of diarrhea and this may support suggestions that some ETEC can be present in the gastrointestinal tract in the absence of a disease state (Smith, 1963; Sojka et al., 1960). The current PCR-based test would not be appropriate to quantify concentrations of ETEC in the gastrointestinal tract. Furthermore, this method of detection does not attempt to identify the toxins produced by K88 *E. coli*. The literature suggests that virulence factors of K88 *E. coli* may be lost over long storage periods (Nagy et al., 1977) or from the repeated culturing of bacteria in an *in vitro* environment (Wilson and Francis, 1986), possibly explaining why the K88ab variant was not detected with the ELISA or Western Blot analyses. It is also possible that the K88ab variants tested were not expressing sufficient fimbria for detection by ELISA or Western Blot. However, upon

conducting ELISA and Western Blot analysis, a determinants were detected from all 3 antigenic variants K88ab, K88ac, and K88ad (original identification confirmed by PCR) (Figure 6). In addition, the K88ad variant showed 100% agreement for all 3 tests; PCR-based, ELISA, and Western Blot. The K88ac variant 08:3030-2 also showed agreement for all 3 tests. Therefore, we conclude that because the a determinant was detected for all K88ab isolates and none of the K88ab isolates tested positive by ELISA or Western Blot for the c or d determinants, that isolates determined as K88ab by our PCR test were indeed of the K88ab variant. Furthermore, primers specific for K88ab isolates did not amplify either the ac or ad isolates, providing further evidence that these isolates were K88ab. Because it has been suggested that the presence of plasmids for hemolysin and fimbrial adhesins (Figure 7) of K88 *E. coli* show some correlation (Smith and Linggood, 1971), we chose hemolytic colonies from challenge studies, when present on blood agar plates, to increase the likelihood of finding adhesive bacteria. The present study found the presence of K88 adhesin fimbrial genes in isolates that lacked hemolysis activity on blood agar plates, thus confirming other workers



Lane 1 shows the DNA standard used for comparison. Lane 2 shows an amplification product using primers AM005 and AM006. Lane 3 shows an amplification product using primers AM005 and MF007. Lane 4 shows an amplification product using primers AM005 and MF008. Lane 5 shows an amplification product using primers AM005 and MF009.

Figure 6. Photograph of PCR products showing all 3 K88 variants, K88ab, K88ac, and K88ad.

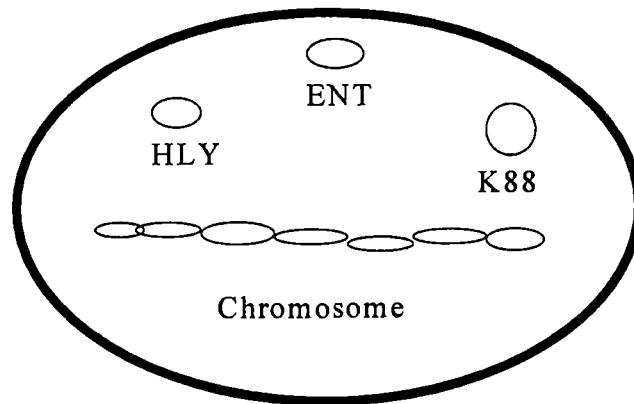


Figure 7. Schematic representation of the genomic makeup of pathogenic *E. coli* showing the chromosomal DNA as well as the plasmids for hemolysin, enterotoxin, and K88.

suggestions that the hemolysis plasmid is not correlated with the fimbrial plasmid (Wilson, 1986). The results of this study indicate that PCR-based testing has several advantages over current methods to differentiate between antigenic variants of K88 *E. coli*. Factors common to SAT, ELISA, and IFAT include the need for antisera, sufficient antigen concentration, and determination of appropriate time and temperature of incubation (Mullaney, et al ., 1991). The PCR-based technique is not dependent upon antigen-antibody reactions and thus no intermediate hosts are needed for the production or titration of antisera which may require a lengthy and tedious procedure. PCR-based testing lends itself to a more rapid analysis and to greater specificity than the simple agglutination test. The sacrifice of animals is also not required for this procedure, which is both a costly and time consuming step. PCR has been reported to enable detection of as little as one genomic copy (Hayashi, 1994), thus making this a practical test for detection of low levels of the pathogen (Figure 8). Through PCR analysis of fecal swabs, a convenient clinical method of detecting K88 *E. coli* now exists. With the previously mentioned advantages, the

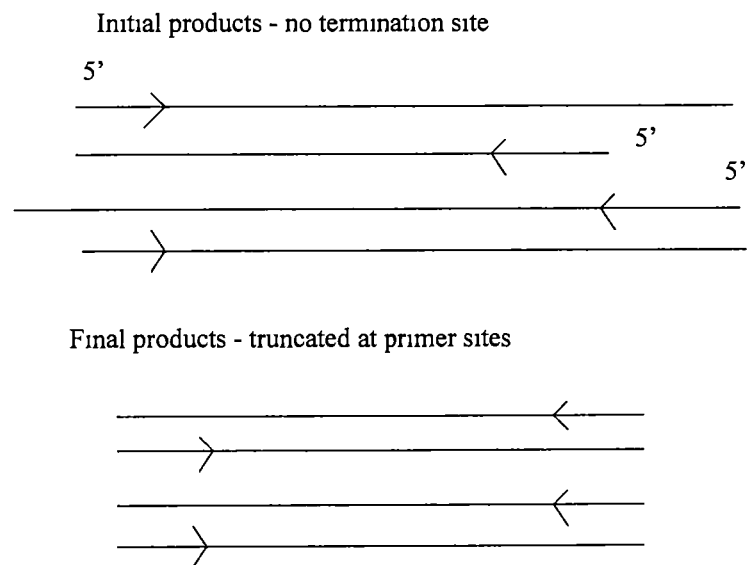


Figure 8. Schematic representation of the initial and final steps in a polymerase chain reaction

detection of K88 *E. coli* by PCR may lead to the more direct diagnosis of the specific organism causing diarrhea in weanling pigs.

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VITA

Mark Ashburn Franklin was born July 29, 1971, and is the son of James Dale and Evetta Franklin. He received his elementary and secondary education in Dandridge, Tennessee, graduating from Jefferson County High School in May of 1989. He then entered the University of Tennessee, Knoxville majoring in Animal Science. Mark graduated from the University of Tennessee with a B.S. in Animal Science in May, 1994. He returned in June of 1994 to the University of Tennessee to begin his graduate work. Mark received his Master of Science with a major in Animal Science in May, 1996.