ADHESION OF K99-POSITIVE ENTEROPATHOGENIC *ESCHERICHIA COLI* TO PIG INTESTINAL BRUSH BORDERS

By

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ABSTRACT

ADHESION OF K99-POSITIVE ENTEROPATHOGENIC
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A study of 242 pigs was performed to determine the incidence of intestinal receptors for K99-positive *Escherichia coli* and to determine if an inheritance pattern existed for the receptors. Ileal samples, collected from pigs at three Michigan slaughterhouses, were examined using a brush border procedure modified for the identification of receptors for K99 pili.

Adhesion of bacteria to brush border fragments indicated the presence of receptors. Receptors were demonstrated in 230 (95%) of the intestinal samples. From zero to greater than ten bacteria adhered per fragment. Following storage of brush borders at 4° C, bacterial aggregates lacking identifiable fragments were present in samples tested for adhesion, suggesting the release of K99 receptors from brush border membranes.

Results from the brush border adhesion test indicate that most, if not all, pigs have receptors for K99 pili. Therefore, it appears that an inheritance pattern similar to that observed for K88 receptors does not exist for K99 receptors.

DEDICATION

To my parents

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INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) is one of the most important causes of neonatal diarrhea in pigs, accounting for approximately 50% of the porcine enteric diseases present during the preweaning period. Enteric colibacillosis is responsible for the loss of millions of dollars in the pig raising industry yearly.

Two virulence determinants are necessary for E. coli to be enteropathogenic: 1) enterotoxin production and 2) pilus production. Three antigenically distinct pili, designated K88, K99 and 987P, are present on separate strains of E. coli capable of infecting pigs.

The pili are filamentous organelles present on the surface of the bacterium. Pili allow the bacteria to attach to receptors specific for the pili on the mucosal surface of the pig's small intestine. Bacteria are then able to colonize the small intestine. Once attached, E. coli produce enterotoxins which lead to hypersecretion, diarrhea, dehydration and often death.

Current research has shown that receptors for the K88 pilus are genetically determined. Previously, it has been suggested that all pigs are susceptible to, or possess receptors for, K99-positive strains of E. coli. However, no substantial work has been performed to determine the

incidence of receptors for the K99 pilus in pigs. Moon et al. (1979) stated that they had not encountered pigs congenitally resistant to colonization by E. coli carrying the K99 pili. They did not indicate how this determination was made. Therefore, it is important to clarify whether or not all pigs have receptors for K99-positive strains of E. coli or if there is a possible inheritance pattern.

The objectives of this study were:

1. To adapt and modify the brush border procedure described by Sellwood et al. (1975) for the identification of intestinal receptors for K99-positive E. coli.
2. To determine the incidence of receptors for the K99 pilus in Michigan swine.
3. To determine whether or not an inheritance pattern may exist for receptors for the K99 pilus.

LITERATURE REVIEW

Since 1899, when *E. coli* was first thought to be a cause of diarrhea in neonatal pigs (Jensen, 1948), an extensive amount of literature has been written about numerous aspects of ETEC infections in swine, as well as calves, lambs and human beings. Several general reviews concerning colibacillosis in pigs and other animals have been published (Gaastra and de Graff, 1982; Kohler, 1972; Nielsen *et al.*, 1968; Rutter, 1975). Emphasis in this literature review will be placed on the most important facts and the details relevant to the present research.

E. coli, a gram negative, motile or nonmotile, nonspore-forming rod, belonging to the family *Enterobacteriaceae*, is a normal inhabitant of the lower intestinal tract of all warm blooded animals (Bruner and Gillespie, 1973). However, when pathogenic strains of *E. coli* are present in the small intestine in large numbers, diarrhea occurs.

Clinical Signs

Piglets from 12 hours of age to several weeks of age, with a peak incidence occurring at three days of age, are susceptible to enteric colibacillosis. Usually several or all the pigs in a litter are affected. Pigs farrowed by

first-litter gilts have a greater incidence of enteric colibacillosis than do pigs farrowed by sows (Blood et al., 1979; Wilson, 1981).

Infected piglets have yellowish or brownish feces which vary from a pasty to a watery consistency. The animals have rough hair coats and normal to subnormal temperatures. They are also anorectic, depressed and weak. As the condition progressively worsens, the animals become dehydrated and emaciated. Pigs commonly die within 24 hours after the onset of clinical signs (Blood et al., 1979; Wilson, 1981).

Lesions

Gross lesions are minimal. The intestines of the affected pigs may be normal or hyperemic, flaccid and distended with fluid or gas. Clotted milk may be present in the stomachs. Catarrhal enteritis may also be present (Blood et al., 1979; Jubb and Kennedy, 1970).

Histologically, few lesions are present in the intestines. Smith and Jones (1963) reported that no inflammatory changes were present in the intestinal tracts of pigs. Christie and Waxler (1972) reported that a mild neutrophilic infiltration of the intestinal villi, hydropic degeneration in villous epithelial cells and edema were present in gnotobiotic pigs infected with E. coli. Bacilli may be seen attached along the margins of the small intestinal villi (Wilson, 1981).

Pathogenesis

Following oral ingestion of E. coli, two important factors are required for the organism to be enteropathogenic. The necessary virulence attributes are: 1) pilus production and 2) enterotoxin production.

Pili

Pili, a Latin term for hairs or hair-like structures, are filamentous organelles projecting from the bacterial surface. Brinton first used the term in association with these structures in 1959. Prior to that time, Duguid et al. (1955) had used the term fimbriae, meaning fringe, threads or fibers in Latin. Both terms are presently being used in the literature.

Two important functions are associated with pili: 1) they increase the active surface area which may facilitate membrane associated activities such as respiration and nutrient uptake and 2) they act as attachment organelles (Ottow, 1975). The latter function plays an important role in the pathogenesis of neonatal enteric colibacillosis.

Structurally, the pili present on ETEC are composed of numerous repeating protein subunits (Isaacson, 1977; Mooi and de Graaf, 1979). The pili on ETEC were originally thought to be capsular (K) antigens and were designated K88 and K99. However, K antigens are polysaccharide in nature and pili are protein in nature. Hence their designation as K antigens is not entirely correct. Pili acting as adhesive

organelles are peritrichously arranged on the bacterial surface (Ottow, 1975).

The pili present on ETEC are distinctly different from the common or type I pili usually associated with E. coli. Several properties of the two types of pili differ. One major distinguishing property is that ETEC pili have mannose-resistant hemagglutinating activity, compared to the mannose-sensitive hemagglutinating activity of type I pili (Duguid et al., 1955; Evans et al., 1979).

Following entry of bacteria into the small intestine, there are several non-immunological defense mechanisms, such as gut peristalsis, villous pumping and flowing ingesta and mucus, which tend to rapidly wash bacteria out of the small intestine (Dixon, 1960; Moon, 1980). Pili, however, enable ETEC to overcome these natural defense mechanisms by allowing bacteria to attach and rapidly proliferate in the small intestine to numbers comparable to those normally found in the sluggishly motile, avillous large intestine (Bertschinger et al., 1972; Jones and Rutter, 1972).

Evidence supporting the fact that pili on ETEC facilitate adhesion has been demonstrated in vivo and in vitro. Microscopically, several early reports demonstrated the close association of ETEC with small intestinal villi (Arbuckle, 1970, 1971; Drees and Waxler, 1970; Moon et al., 1971; Staley et al., 1969). Bertschinger et al. (1972) showed that enteropathogenic E. coli (EEC) are more likely to be associated with the intestinal epithelium than nonenteropathogenic E. coli (NEEC). The EEC were present

adjacent to the brush border along the entire villus, but the NEEC were randomly distributed in the central lumen.

In conventionally reared pigs, Jones and Rutter (1972) demonstrated that the K88-positive strain of E. coli was able to adhere to the mucosa of the small intestine and cause diarrhea, compared to the K88-negative mutant which was unattached, was distributed throughout the lumen and did not cause diarrhea. The results indicated that the K88 pilus is responsible for adhesion of K88-positive bacteria to the small intestinal mucosa and that adhesion is required for the virulence of K88-positive bacteria in pigs. Supportive evidence for pili facilitating adhesion was also provided by Nagy et al. (1976, 1977) when strain 987P, a K88-negative piliated strain of ETEC, was also shown to adhere to the small intestine.

The in vitro adhesion of piliated ETEC has been demonstrated with the brush border technique (Sellwood et al., 1975), intestinal epithelial cell technique (Isaacson et al., 1978a; Nagy et al., 1977; Wilson and Hohman, 1974), the intestinal villous technique (Girardeau, 1980) and hemagglutination studies (Evans et al., 1979).

The pili most commonly present on strains of E. coli capable of causing diarrhea in neonatal pigs are K88, K99 and 987P. Recently, adhesive factors distinct from the former three, with characteristics of mannose-resistant pili, have been discovered (Aning and Thomlinson, 1983; Awad-Masalmeh et al., 1982; Moon et al., 1980). Morris et

al. (1983) have recently discovered the F41 pilus on piglet strains of ETEC. The F41 pilus appears to be the same pilus that was described by Moon et al. (1980) and Awad-Masalmeh et al. (1982). Two strains of ETEC have been shown to produce both K88 and K99 pili (Schneider and To, 1982).

The K88 Pilus. Of the three most commonly found pili, the K88 pilus was the first to be discovered and consequently has had the most written about it. Ørskov et al. first described the K88 pilus in 1961. The extrachromosomally transferred K88 pilus is encoded for by a 50 megadalton plasmid (Ørskov et al., 1966; Shipley et al., 1978). Chemically, the K88 pilus has been identified as a protein, containing all the common amino acids except cysteine-cystine, with little or no carbohydrate present (Anderson et al., 1980; Stirm et al., 1967b). The lack of sulfur-containing amino acids suggests that no disulfide bonds are present in the pilus. Structurally, Stirm et al. (1967a) described it as a filamentous antigen with a diameter of 7-11 nanometers (nm) and a length of 100 to 150 nm. Wadström et al. (1979) estimated the K88 pilus's diameter to be 2.1 nm. The K88 pilus is a polymer of noncovalently linked subunits whose monomeric molecular weight ranges from 23,000 to 26,000, depending upon the serologic variant (Anderson et al., 1980; Mooi and de Graaf, 1979).

Serologically, there are three variants of the K88 antigen, K88ab, K88ac and K88ad (Guinée and Jansen, 1977a; Ørskov et al., 1964). The common antigenic determinant, a, is combined with a variable antigenic factor designated b, c

or d. The existence of the K88ab and K88ac variants has been known since 1964, but the K88ad variant was just discovered recently. The variants are antigenically different due to amino acid sequence differences in the pilus proteins. Amino acid sequences corresponding to the antigenic variables a, b, c and d have been predicted (Gaastra and de Graaf, 1982; Gaastra et al., 1979, 1983; Klemm and Mikkelsen, 1982).

The K88 pilus is species specific. Strains of E. coli that are K88-positive are able to colonize pig intestine in vivo and cause diarrhea, but K88-positive ETEC have not been reported as enteropathogenic in other species (Moon et al., 1977; Smith and Halls, 1967).

The 987P Pilus. Present knowledge about the 987P pilus is limited. The 987P pilus was first determined to facilitate intestinal adhesion and colonization in 1976 (Nagy et al., 1976). The pilus is composed of protein and has a diameter of 7 nm (Fusco et al., 1978; Isaacson and Richter, 1981). The molecular weight of the 987P pilus's subunit was first reported as 18,900 by Fusco et al. (1978) and has since then been reported to be 20,000 by Isaacson and Richter (1981). Presently, it is not known whether or not the 987P pilus is genetically encoded for by a plasmid or a chromosome (Moon et al., 1979). The 987P pilus is also considered to be species specific as a virulence factor in pigs, even though it has been found on calf strains of E.

coli. In calves, 987P piliated strains of E. coli appear to be harmless (Moon, 1978b).

The K99 Pilus. Ørskov et al. first described the Kco antigen present on calf and lamb enteropathogenic strains of E. coli as K99 in 1975. In 1977, the K99 pilus was discovered on pig strains of ETEC (Moon et al., 1977). Isaacson (1977) concluded that the K99 antigen was a pilus based on its subunit structure, external surface location and rod-like shape (1977). The K99 pilus is encoded for by a transmissible plasmid (Smith and Linggood, 1972). The pilus's length was reported to be 130 to 160 nm and its diameter was reported to be 7.4±.6 nm (Altmann et al., 1982; Isaacson, 1977). Recently, Isaacson et al. (1981) have reported the pilus's diameter to be 4.8 nm.

The K99 pilus is protein in nature and lacks the sulfur-containing amino acids, cysteine and methionine (Altmann et al., 1982). In the past, there has been a great deal of controversy concerning the molecular weight and characterization of the K99 pilus with disparate reports based on different isolation procedures originating from several laboratories (Altmann et al., 1982; Chantner, 1982; de Graaf et al., 1981; Isaacson, 1977, 1978; Morris et al., 1977, 1978a,b). Recently, Morris et al. (1980) have reported that there are two pilus subunits present in serogroups 09 and 0101 with different molecular weights and ionic charges. The cationic and lower molecular weight component is the K99 pilus and the high molecular weight, anionic component is F41 which has been shown to be a pilus

with adhesive abilities (Morris et al., 1982). The molecular weight of the K99 pilus subunit was reported by de Graaf (1981) to be 18,500 and by Chanter (1982) to be 19,000.

The K99 pilus is not species specific. Calf, lamb and pig strains of E. coli bearing K99 pili are pathogenic (Moon et al., 1977; Ørskov et al., 1975; Smith and Linggood, 1972).

Receptors

Pili let bacteria attach to special receptors present on the small intestinal mucosa. Present knowledge about the actual nature of the receptors to which K88, K99 and 987P attach is limited. Research is currently ongoing to determine the chemical composition of the receptors. Reports indicate that chemically the receptor is composed of glycolipids or glycoproteins (Gibbons et al., 1975; Kearns and Gibbons, 1979). Gibbons et al. (1975) determined that glycoproteins with a terminal β-D-galactosyl structure were able to inhibit the hemagglutination of guinea pig erythrocytes by K88 pili, suggesting that the glycoprotein might bind with the K88 pilus and inhibit hemagglutination due to its resemblance to the K88 receptor. The β-galactosyl residues have also been shown to play an important role in the binding of K88 pili to brush borders (Sellwood, 1980). Binding of K88 pili to brush borders occurs from 4° C to 37° C, while binding to the guinea pig erythrocytes only occurs at 4° C, indicating that the fit for the natural receptors is better than that of the guinea pig erythrocyte receptors

(Sellwood, 1980; Gibbons et al., 1975). Glycolipids have also been shown to have K88 receptor activity (Sellwood and Kearns, 1979; Kearns and Gibbons, 1979). The brush border receptor for the K88 pilus reacts chemically in a way similar to that of the cholera toxin receptor which is a G_{M1} ganglioside; however, according to Sellwood and Kearns (1979), it doesn't really appear to be a ganglioside.

Sugars and glycoproteins inhibit binding to brush borders. Glycoproteins with N-acetylglucosamine and N-acetylgalactosamine as terminal sugars inhibit binding of the K88 antigen and, to a lesser degree, sugars alone, such as N-acetylglucosamine, N-acetylgalactosamine and N-acetylmannosamine, can also inhibit binding (Anderson et al., 1980).

Hemagglutination tests have been performed on erythrocytes from several species in an attempt to identify specific receptor sites. Parry and Porter (1978) demonstrated that K88ab adhered strongly to chicken erythrocytes but not to the extent of that seen on the brush borders of intestinal epithelial cells. Guinea pig erythrocytes were agglutinated by both K88ab and K88ac pilus preparations and live organisms. They concluded that both the a and the b or c determinants of the K88 pilus for K88ab and K88ac are involved in adhesion to intestinal epithelial cells and guinea pig erythrocytes. However, in the chicken erythrocyte, adhesion of the pilus appears to involve only the K88b determinant's receptor on the erythrocyte (Parry and Porter,

1978). The hemagglutination reaction is easier to study, but the adhesion is not as specific as that seen with the natural receptors on epithelial cells.

Intestinal epithelial cells are believed to have different receptors for different pili. Using strains of E. coli with K99 and 987P pili, binding of piliated E. coli to brush borders was inhibited by homologous pili, but not heterologous pili which indicated that a difference in receptors existed (Isaacson et al., 1978a).

There may not be a separate receptor for each of the serologic variants of K88-positive E. coli. Bijlsma et al. (1982) showed that blocking the receptor site with one serologic variant of the K88 antigen also inhibited the adhesion of other K88 variants. For example, when brush borders from pigs susceptible to all three serologic variants of E. coli were exposed to the K88ab antigen and the receptor sites were blocked, then K88ad- and K88ac-positive strains of E. coli were no longer able to adhere. However, adhesion of K88ad to brush borders from one pig susceptible to all three serologic variants of K88 was not inhibited by K88ab or K88ac antigen. This suggests that the receptor site for K88ad in brush borders from pigs susceptible to all three serologic variants may have two configurations which can only be detected by receptor blocking. Bijlsma et al. consider that only one receptor, depending upon its modifications, will allow attachment of one, two or all three of the K88 serologic variants (Bijlsma et al., 1982).

Wilson and Hohmann (1974) believe that the K88a antigenic component doesn't affect the ability of bacteria to attach to intestinal mucosal cells. They found that the antigen necessary for blocking adhesion was the c or b component. Bijlsma *et al.* (1982) say that it is unlikely that the antigenic components b, c or d are exactly the same as the adhesion component of the K88 pilus because K88ac antigen is able to block K88ab and K88ad receptors of phenotype A brush borders, yet K88c antibody doesn't react with K88b and K88d. Conversely, Parry and Porter (1978) were able to block the adhesion of K88ab- and K88ac-positive strains of *E. coli* to brush borders by using antisera specific for the K88a determinant. Antibodies against K88b and K88c antigens only inhibited homologous strains of K88-positive *E. coli*.

It appears that receptors for pili are capable of releasing *in vitro* from brush borders or intestinal cell membranes. Kearns and Gibbons (1979) reported that plasma membranes prepared from positive brush borders lost 98% of their receptor activity. They were able to demonstrate that a supernatant fraction, obtained during the preparation of K88-positive brush borders, was able to enhance the adhesion of K88-positive *E. coli* to both positive and negative brush borders. Supernatant fractions obtained from negative brush borders did not enhance adhesion. Utilizing pig mucosal organ cultures, Staley and Wilson (1983) have demonstrated the release of receptors for K88-positive *E. coli* into culture media. Dean and Isaacson (1982) have identified a

soluble 987P pilus receptor-containing fraction which was released from brush borders stored at 4° C. They have shown that receptor activity is heat stable and that the receptor-containing fraction consists of over 40 proteins.

Enterotoxins

Once attachment and colonization have occurred, ETEC are able to produce enterotoxins. Enterotoxin production is the second important virulence determinant (Smith and Hall, 1967). Enterotoxins are factors that cause the net movement of fluid and electrolytes from plasma to the intestinal lumen when given by the intraluminal route (Dorne et al., 1976). Two types of enterotoxins, based on their thermolability, antigenicity and molecular weight, are produced by ETEC: 1) a heat stable (ST), nonantigenic enterotoxin with a low molecular weight (Smith and Halls, 1967; Smith and Gyles, 1970) and 2) a heat labile (LT), antigenic enterotoxin with a high molecular weight (Gyles, 1974; Gyles and Barnum, 1969). The heat labile toxin is inactivated by heat at 60° C for 15 minutes and the heat stable toxin is resistant to temperatures of 100° C for 15 minutes. Both types of enterotoxin production are controlled by transmissible plasmids (Skerman et al., 1972; Smith and Halls, 1968).

Strains of ETEC may produce only one or both types of enterotoxin. Strains of ETEC that possess the K88 pilus may be associated with the production of LT, ST or both (Guinée and Jansen, 1979; Gyles and Barnum, 1969; Smith and Gyles, 1970; Söderlind and Möllby, 1979). The 987P pilus is

associated with the production of ST (Guinée and Jansen, 1979b; Moon et al., 1980). The K99 pilus is also associated with ST production (Guinée and Jansen, 1979b; Isaacson et al., 1978b; Moon et al., 1976).

Although many different strains of ETEC produce LT, it is a highly homologous substance in all the strains. The LT was considered by Finkelstein et al. (1976) to be a heterogenous molecule whose molecular weight ranged from 35,000 to 100,000 Daltons, but Dorner (1976) and Evans et al. (1976) reported a molecular weight of approximately 100,000 Daltons. Finkelstein et al. (1976) suggested that the heterogeneity was due to proteolytic splitting of the molecule during purification.

The LT is similar, antigenically and functionally, to the cholera toxin of Vibrio cholera (Carpenter, 1972; Clements and Finkelstein, 1978; Gyles, 1974; Gyles and Barnum, 1969; Moon, 1971). Both toxins activate membrane bound adenyl cyclase which consequently leads to an increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP) in epithelial cells of the small intestine (Evans et al., 1972; Hewlett, 1974). Subsequently, there is a net secretion of an isotonic, alkaline fluid rich in electrolytes as a result of increased sodium secretion and decreased chloride absorption. The increased secretion by the crypt cells leads to hypersecretion and a secretory diarrhea (Moon et al., 1978a). Hamilton et al. (1978) have tested LT in pig ligated loops and stated that activation of adenyl cyclase

by bacterial enterotoxins may not be essential for intestinal secretion.

The second type of enterotoxin is ST. The ST is considered to be nonantigenic, although it has been shown to act as a hapten. It may be made antigenic by coupling it to a carrier protein (Alderete and Robertson, 1978; Frantz and Robertson, 1981). However, according to Moon et al. (1983) when pregnant swine are immunized with ST_A coupled to a carrier protein, they are not protected against ETEC producing ST_A alone. The vaccine stimulated the production of antibodies with high binding activity but low neutralizing activity and thus low protective activity for suckling pigs. The ST is a monomeric octadecapeptide (Lallier et al., 1982) with a molecular weight of 4,400 (Alderete and Robinson, 1978).

There is evidence that suggests that STs from different strains of ETEC represent a heterogenous group of E. coli (Guerrant et al., 1975; Steiner et al., 1972). At least two distinct types of ST are known: 1) ST_1 or ST_A which is methanol soluble and active in the infant mouse model and 2) ST_{II} or ST_B which is methanol insoluble and inactive in the infant mouse model (Newsome et al., 1978).

Utilizing various assay systems, ST doesn't appear to activate adenyl cyclase (Giannella, 1977; Hamilton et al., 1978; Sack, 1975; Smith and Gyles, 1970). The mechanism of action of ST involves the activation of guanylate cyclase in the small intestinal epithelial cells (Hughes et al., 1978; Newsome et al., 1978).

Resistance to E. coli Enteric Infections

Neonatal pigs are highly susceptible to diarrhea caused by ETEC. Diarrhea caused by K88-positive ETEC may occur during the neonatal and postweaning period, but diarrhea due to K99-positive ETEC is not known to occur after weaning in pigs three to eight weeks old (Moon, 1978b; Sojka, 1965). As pigs age, they become resistant to ETEC (Moon and Whipp, 1970; Nielsen et al., 1968; Smith and Halls, 1967). The mechanism of age resistance is unknown. British researchers have shown that pigs may be born resistant to diarrhea caused by K88-positive ETEC (Gibbons et al., 1977; Sellwood et al., 1975).

Inherited Resistance to K88-Positive E. coli

British researchers have determined that the presence or absence of receptors for the K88 pilus of E. coli is inherited. Two alleles are present at a single locus and are inherited in a simple Mendelian manner. One allele, the allele coding for the receptor, is dominant over the other allele. Three genotypes occur: homozygous dominant (SS, susceptible, adherent), heterozygous (Ss, susceptible, adherent) and homozygous recessive (ss, resistant, non-adherent). Two phenotypes occur, susceptible and resistant. The susceptible or positive phenotype is the expression of the dominant allele (Gibbons et al., 1977; Sellwood et al., 1975). Phenotypic expression is independent of the age of the pig and is fully established at birth (Gibbons et al., 1977).

It is difficult to study the genetic aspects of E. coli resistance since the phenotype usually cannot be observed until the animal is dead (Gibbons et al., 1977). Recently, though, biopsies by enterotomy have been performed on living animals (Snodgrass et al., 1981). The simple in vitro technique used by Sellwood et al. (1975) demonstrates the adhesion of K88-positive E. coli to brush borders from pig intestinal cells. Adhesion of bacteria to brush borders occurs in pigs which have the receptors (positive pigs) and no adhesion occurs in pigs lacking the receptors (negative pigs) (Sellwood et al., 1975).

With the previous technique, pig litters are divided into three types: 1) non-segregating susceptible (entire litter susceptible), 2) non-segregating resistant (entire litter resistant) and 3) segregating (litter contains piglets of both phenotypes). Gibbons et al. (1977) considered that matings which gave a non-segregating resistant litter of more than 6 piglets were derived from homozygous recessive parents. Once the pigs are conditionally identified as homozygous recessive animals, they are mated to unknown animals and the resulting litter is phenotyped. The unknown sire or dam is then assigned a provisional genotype and phenotype. If the litter segregates, then the unknown parent is a heterozygote. If the litter doesn't segregate and all piglets are susceptible, then the parent is homozygous dominant. Or, if the litter doesn't segregate and all piglets are resistant, then the parent is

homozygous recessive. Therefore, genotypes and phenotypes can be assigned to the parents (Gibbons et al., 1977).

Rutter et al. (1973) have shown experimentally that negative pigs are resistant to infection by K88-positive E. coli. K88-positive bacteria colonized the gut of positive pigs more readily than the gut of negative pigs, and the positive piglets were more likely to be susceptible to diarrhea caused by E. coli. In negative piglets, the organisms were unable to attach and rapidly disappeared from the intestines (Rutter et al., 1973). Sellwood (1979) reported that in a natural outbreak of scours, negative piglets were resistant to diarrhea caused by E. coli. However, susceptibility to infection is a little more complicated in an in vivo situation. Positive piglets receiving colostrum or milk from the dam that contains K88 antibodies are passively protected (Rutter and Jones, 1973). It is assumed that positive piglets are susceptible to infection, but passive protection due to K88 antibodies in the colostrum prevents enteropathogenic E. coli from attaching and multiplying to high numbers in the small intestine. The negative phenotype primarily determines whether an animal will be resistant to infection with K88-positive strains of E. coli, but if the positive animals receive antibodies, they may also be resistant to infection (Rutter et al., 1973).

In herds examined by Gibbons et al. (1977), both genes were present with S predominating. The S gene, or susceptible gene, is at a disadvantage to the s gene or resistant

gene since the environment contains virulent K88-positive *E. coli*. There are many explanations for the persistence of the S gene, some are based on compensatory selection against the homozygous recessive genotype. However, there is really no substantial evidence for compensatory selection. No statistically significant differences between resistant and susceptible animals with respect to the economically important parameters of growth rate, food conversion efficiency and carcass quality can be shown. There is also no reason to suspect artificial selection by the breeder. The explanation proposed is that under normal conditions, piglets are protected from neonatal colibacillosis by antibodies in the colostrum (Gibbons *et al.*, 1977).

In the course of an epidemic, the dam becomes immune, and the selective advantage of the ss genotype becomes reduced. In the initial stages of the disease, when virulent K88-positive *E. coli* are introduced into a herd not previously or recently exposed to the organism, there is a high selection in favor of the resistant gene. The susceptible breeding sows begin to supply antibodies in their colostrum, but the resistant sows don't recognize K88-positive bacteria as a pathogen and hence antibody synthesis is not stimulated. Once immunity is established in a herd, the offspring of both homozygous dominants and heterozygotes will be well protected against colibacillosis. The offspring of homozygous recessives are protected only if they are themselves homozygous recessives, so the selective

advantage of the recessive homozygote is reduced. The heterozygous offspring of homozygous recessive dams are at a considerable disadvantage compared to the rest of the pig population. They are genetically susceptible and minimally protected by antibodies in the colostrum or milk. Thus, there is a selection against a particular class of heterozygotes, which tends to eliminate the less common gene (Gibbons et al., 1977).

Most of the studies on the genetic aspects of receptors for K88-positive E. coli have been done on pigs in England and Australia. Only a few studies have been performed in the United States. In Michigan, Sher and Waxler (1981a,b) randomly collected intestinal samples from the Michigan State University Department of Animal Science swine herd at the time of slaughter and examined them for K88 receptors by the brush border adhesion technique. Using a strain of E. coli carrying the K88ac pilus, 23% of the 109 pigs tested had receptors. When a similar study was carried out by Waxler et al. (1983), using 14 litters sired by four boars from the same herd, 38% of the pigs had receptors. The results found by Sher and Waxler (1981a,b) and Waxler et al. (1983) suggest that the percentage of pigs with K88 receptors in Michigan is lower than that reported in England (Sellwood et al., 1975) and Australia (Snodgrass et al., 1981).

Recently, Bijlsma et al. (1982) in the Netherlands have found that there are five phenotypes in swine based on the three serologic variants of the K88 antigen.

Table 1. Pig phenotypes based on K88 serologic variants of Escherichia coli.

Phenotype	Adhesion of E. coli strains producing:		
	K88ab	K88ac	K88ad
A	+	+	+
B	+	+	-
C	+	-	+
D	-	-	+
E	-	-	-

The phenotypes differ depending upon whether or not a pig is susceptible to adhesion in the brush border test to three (phenotype A), two (phenotypes B and C) or one (phenotype D) of the serologic variants of K88 or resistant to all three variants (phenotype E). Sellwood's adhesion negative phenotype corresponds to the D and E phenotypes, because the K88ad serologic variant of E. coli was not discovered when he performed his study. The existence of five phenotypes complicates the rather simple genetic model proposed by British researchers and additional work is needed to determine the inheritance pattern of the five phenotypes.

In the future, it may be possible to reduce the level of neonatal scours caused by K88-positive E. coli in a herd which has not been genotyped by using only resistant boars when breeding sows. Thus, susceptible offspring can only be

produced by susceptible dams capable of protecting them by antibody production. Progeny born of resistant dams will produce resistant offspring which will not require maternal protection. There should be an immediate decrease in neonatal scours. With the continued use of resistant boars, the incidence of the resistant genotype should become increasingly common. Genetic protection will then be possible as compared to passive protection (Sellwood, 1979). Biopsy by enterotomy may allow the pig farmer who is reluctant to buy boars of the negative phenotype, which may not meet the standards of conformation or performance of his herd, with the alternative of identifying negative animals within his existing breeding stock (Snodgrass et al., 1981).

Resistance to K99-Positive E. coli

Moon et al. (1979) have stated that they have not encountered pigs congenitally resistant to colonization by E. coli carrying the K99 or 987P pili. However, using isolated small intestinal epithelial cells from pigs one day, three weeks and six weeks old, Runnels et al. (1980) demonstrated resistance with age to K99-positive E. coli. From 8.8 to 14.5% more K99-positive ETEC were shown to adhere to one-day-old piglet epithelial cells than to six-week-old pig epithelial cells. The resistance to adhesion wasn't demonstrable with 3-week-old pigs.

Culture Procedures for K99-Positive E. coli

In vitro studies with K99-positive and 987P-positive ETEC have been hampered by the extreme difficulty in growing and detecting piliated organisms in culture media (Guinée et al., 1976; Isaacson et al., 1978a; Ørskov et al., 1975). In contrast, K88-positive ETEC become piliated relatively easily in several types of ordinary media, such as trypticase soy broth, under routine growth conditions. Recently, progress has been made in the development of new media and culture procedures which enhance the production and detection of piliated E. coli in vitro. Several factors, such as temperature, oxygen concentration, glucose concentration and amino acid concentration, are capable of influencing the expression of the K99 pilus.

The expression of the K99 pilus, and other pili, is temperature dependent. The optimum temperature for pilus production is 37° C (Brinton, 1965; de Graaf et al., 1980b; Isaacson et al., 1977; Ørskov et al., 1975). Below 30° C, no significant levels of pili are detectable (de Graaf et al., 1980b). At 18° C, pilus production does not occur (Brinton, 1965; Isaacson et al., 1977; Ørskov et al., 1975). Both the subunit synthesis and pilus assembly of the K99 pilus are inhibited at 18° C (Isaacson, 1983).

Pilus production is also affected by the oxygen concentration. Under anaerobic conditions, the production of K99 is severely inhibited. Isaacson (1980) noted that when aeration is enhanced due to mechanical shaking, there

is a six-fold increase in K99 production, while nonaerated bacteria produce essentially no K99. Generally, the intestine is considered to have a low reduction-oxidation potential, so, as postulated by Girardeau et al. (1982b), either special growth conditions are present in the intestine which enable E. coli to produce K99 pili at a low reduction-oxidation potential or those bacteria which are attached to the intestinal mucosa are in a relatively aerobic environment in comparison to that of the central lumen.

Several ideas concerning why K99 is so difficult to detect have been proposed. Guinée et al. (1976) suggested that the K99 antigen was masked by the abundant capsular antigen or that the enzymes involved in K99 synthesis were inhibited by catabolites of glucose. According to Isaacson et al. (1978b), passing K99-positive ETEC in liquid medium several times leads to the development of acapsular mutants and a corresponding increase in the detectability of K99. However, Girardeau et al. (1982b) has observed no association between K99 detection and the absence or presence of capsular antigens.

Isaacson (1980) reported that glucose repressed K99 synthesis, especially at a concentration of 0.5% glucose. However, when cyclic adenosine 3',5'-monophosphate was added to the medium, the glucose mediated repression of K99 synthesis was overcome. The greatest expression of the K99 pilus occurred at a 5 mM concentration of cAMP. In contrast to what Guinée et al. (1976) had found previously, Isaacson (1980) determined that glucose repression was indeed subject

to cAMP-dependent catabolite repression. The difference in results was probably due to the much lower concentration of cAMP used by Guinée et al. (1976) compared to the concentration used by Isaacson (1980).

Using M_2 Minca medium with ammonium sulfate as the nitrogen source, the expression of the K99 pilus in two ETEC strains was not repressed by a high (2%) concentration of glucose. Twelve strains of bacteria were tested by Girardeau et al. (1982b). Two different groups, based on their glucose dependency, were found: one group where K99 pilus production occurs on Minca medium without glucose and a second group where K99 is produced only in the presence of glucose.

Isaacson (1983) has recently reported that the addition of glucose to the growth media doesn't affect K99 subunit synthesis or pilus assembly. The glucose-dependent expression of K99 is sensitive to the reduction-oxidation potential. Glucose shows a greater inhibitory effect on K99 production when the bacteria are grown under anaerobic conditions instead of aerobic conditions (Girardeau et al., 1982b).

The amino acid L-alanine has an inhibitory effect on K99 synthesis (de Graaf et al., 1980a). Concentrations of L-alanine greater than 8 mM lead to more than 80% inhibition of K99 synthesis in media rich in amino acids (Girardeau et al., 1982a). Adding L-threonine or L-isoleucine to the media decreases the effects of L-alanine (Isaacson, 1983).

Girardeau et al. (1982a) believe that L-alanine is inhibitory to K99 synthesis due to its involvement in either enzyme repression or the inhibition of plasmid expression. The reduction-oxidation potential is also important since there was a greater inhibitory effect by L-alanine on K99 synthesis when bacteria are grown under anaerobic rather than aerobic conditions (Girardeau et al., 1982a).

Since K99 is usually not detectable on ETEC grown at 37° C on routine culture media designed for the isolation of Enterobacteriaceae (Guinée et al., 1976), several types of synthetic media have been developed to enhance the production of the K99 pilus antigen. In complex media, the production of K99 is strongly decreased. Minca, a buffered semi-synthetic medium at pH 7.5, improves the detectability of the K99 antigen, especially when the cultures are subcultured two or three times in liquid Minca. Cultures strongly agglutinate with K99 antiserum on primary isolation, but there is only weak agglutination following subculturing on solid Minca (Guinée et al., 1976). When cultures are grown on solid Minca at 37° C for only 6-8 hours, the detection of K99 is improved. IsoVitaleX added to Minca medium helps to improve the detection of K99, possibly by stabilizing the K99 pilus on E. coli. In solid Minca, the pili may become stuck to the bacterial surface, but in liquid Minca, the pili are more stretched or rigid which enhances their detectability. Therefore, it is beneficial to pass E. coli through liquid medium. Detectability of the K99 antigen is improved when cultures are grown for 6-8 hours or 20-24

hours on Minca-IsoVitaleX (MI) medium also (Guinée et al., 1977).

Frances et al. (1982) have found that E medium, a synthetic medium containing essential salts, citric acid and 1% dextrose, is better than blood agar, MI agar, MI broth and TSB (without glucose) for increasing the detectability of K99-positive E. coli from diarrheic calves and piglets. They have recommended that several media, such as blood agar, E agar and MI broth, be used when culturing ETEC for pilus typing to enhance the chance of detecting pili. The E agar, according to Frances et al. (1982), is less expensive, is less chemically complex and has a much more luxuriant growth pattern for K99-positive E. coli than MI agar.

E broth is nearly as effective as MI broth in promoting 987P production. It is not as effective as blood agar, though, in promoting the expression of K88 (Frances et al., 1982). Blood agar promotes the expression of many K88- and 987P-positive strains (Moon et al., 1980).

Isaacson (1983) reported that the synthesis of K99 subunits occurs throughout the life cycle of the bacterium and assembled K99 pili appear during the logarithmic phase. Approximately 92% of all cellular K99 is associated with the outer membrane, and 4% is associated with the inner membrane. No K99 is detected in the cytoplasm.

Pilus vaccines

Currently, several pilus vaccines have been developed for the protection of pigs against neonatal enteric colibacillosis. Vaccines are administered parenterally or orally to sows prior to farrowing (Moon, 1981). The vaccine induces protective levels of antibody in the sow's colostrum and milk which provide passive immunity to the newborn pig. Research has demonstrated that protection correlates with antipilus antibodies in the colostrum and milk (Acres et al., 1978; Morgan et al., 1978; Nagy et al., 1978; Rutter and Jones, 1973; Rutter et al., 1976). Several researchers believe that colostral antibodies prevent the colonization of ETEC by blocking adhesion (Nagy et al., 1978; Rutter, 1975; Rutter et al., 1973, 1976). Piglets suckling vaccinated dams are more resistant to infection by ETEC than control pigs when challenged by ETEC with homologous pili (Moon, 1981). According to Nagy et al. (1978), mortality, morbidity and the duration of diarrhea are decreased in piglets suckling vaccinated dams. Also, the number of E. coli attached to the villous epithelium of the small intestine is decreased.

Several vaccines which use either purified pili or whole bacterial cell preparations have been developed. Pigs may be infected by strains of ETEC with different pilus antigens, so multivalent vaccines are necessary to provide protection from enteric colibacillosis. Pitman-Moore has developed a trivalent bacterin called Porcimune which

contains K88, K99 and 987P piliated strains of E. coli (Anonymous, 1981). Recently, Pitman-Moore has also developed an E. coli bacterin containing 4 piliated strains of E. coli, K88, K99, 987P and F41 (To, 1983).

Subunit vaccines, consisting of pili only, have also been developed. A trivalent subunit vaccine which utilizes recombinant DNA gene splicing techniques has been made by Salsbury. The plasmids responsible for pilus production are introduced into a laboratory strain of E. coli. The laboratory strain of E. coli then produces many more pili than are normally produced by a wild strain of E. coli. The pili are sheared off from the bacterial surface by mechanical means and incorporated into the vaccine. The endotoxins associated with bacterins, which cause adverse effects such as allergies, shock and abortions, are excluded from subunit vaccines (Anonymous, 1983).

MATERIALS AND METHODS

Specimens

Samples of ileum, each measuring six to eight inches in length, were obtained from 242 freshly killed pigs at local slaughterhouses. The slaughterhouses were Michigan State University Meats Laboratory, East Lansing, MI (1/20/83-8/22/83, 60 specimens), Milligan's Meat Packing Plant, Jackson, MI (7/15/83-8/24/83, 148 specimens) and Bain's Packing Company, Howell, MI (9/7/83-9/14/83, 34 specimens). Ileal samples were carefully flushed with cold 0.15 M NaCl solution to remove mucus and ingesta. They were then placed in plastic bags and packed in crushed ice until further procedures were carried out, usually within two to three hours of the time of slaughter.

Brush Border Preparations

Intestinal specimens were processed using a modification of the procedure described by Sellwood *et al.* (1975). In the laboratory, the intestinal specimen was ligated on one end, the lumen was filled with an EDTA buffer solution containing 0.096 M NaCl, 0.008 M KH_2PO_4, 0.0056 M Na_2HPO_4, 0.0015 M KCl and 0.01 M ethylenediaminetetracetate (EDTA), pH 6.8 and the other end of the intestine was clamped with a

hemostat. The buffer was at room temperature. The intestinal specimen was filled with the buffer until slight distention occurred. To prevent drying of the external surface, sections of ileum were immersed in a similar solution containing 0.3 M sucrose instead of EDTA, pH 6.8, for 15-20 minutes at room temperature. Next, the EDTA buffer in the intestinal lumen was discarded and replaced with the sucrose buffer until the lumen was half-filled. The epithelial cells were detached by gently massaging each intestinal section between the thumb and fingers. The intestinal contents were collected in 50 ml plastic centrifuge tubes. The intestine was again half-filled with sucrose buffer and massaged. The latter procedure was repeated until 45-50 ml of the epithelial cell suspension were obtained.

The following steps were performed at 4^{o} C. The epithelial cell suspension was centrifuged at 1200 x g for 15 minutes[a]. The supernatant was removed from each tube, and the pellet was resuspended to a volume of 45-50 ml with cold 0.005 M EDTA, pH 7.4 (adjusted with 0.5 M Na_2CO_3). Next, the suspension was homogenized with a Teflon serrated-tipped tissue grinder (clearance - 0.15-0.23 mm)[b] by moving the pestle up and down six times while it rotated at approximately 900 rpm. The suspension was centrifuged at 300 x g for 5 minutes, and the supernatant was discarded. The

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[a]International Refrigerated Centrifuge, Model PR-6, International Equipment Co., Needham, MA.

[b]Thomas Co., Philadelphia, PA.

pellet was resuspended with the EDTA buffer, and homogenization and centrifugation were repeated three to five times until the supernatant appeared clear. Next, the pellet was washed three times with Krebs-Henseleit (KH) buffer, pH 7.4, composed of 0.12 M NaCl, 0.014 M KCl, 0.025 M $NaHCO_3$ and 0.001 M KH_2PO_4. The pellet was then resuspended to eight times its volume with KH buffer and filtered through glass wool. The KH solution containing the brush borders was allowed to stand for 15 minutes to let any mucus present rise to the surface. The mucus was discarded, and the brush border suspension was held overnight at 4° C. For prolonged storage, part of the brush border suspension was mixed with an equal volume of glycerol and stored at -70° C (Bijlsma et al., 1982).

E. coli Strains

The K99-positive E. coli used in the brush border adhesion test was strain 431 (0101:K30). The culture, obtained from Dr. R. E. Isaacson, School of Public Health, University of Michigan, Ann Arbor, was maintained in Minca broth (Guinée et al., 1977) with IsoVitaleX[c] (MIB). Cultures of E. coli 431 were aerobically incubated at 37° C in a shaking water bath[d] for 12-16 hours prior to testing for

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[c]Baltimore Biological Laboratories, Cockeysville, MD.

[d]Precision Scientific Co., Chicago, IL.

adhesion. The culture was transferred daily from MIB to MIB to insure piliation on the day of testing.

The K99-negative strain was isolated from a feline urinary culture, and stock cultures were maintained in trypticase soy broth[e] (TSB). The cultures of E. coli in TSB were incubated aerobically at 37° C for 12-16 hours prior to testing.

Following incubation, both cultures were centrifuged at 1500 x g for 20 minutes. The supernatant was discarded, and the bacteria were washed in KH and centrifuged three times. The pellets were then resuspended in 5 ml of KH buffer to a concentration of approximately 1 x 10^9 colony-forming units per ml.

Slide Agglutination

A standard slide agglutination test was carried out to verify piliation using K99 antiserum. Prior to this study, the antiserum was produced in Dr. G. L. Waxler's laboratory, Department of Pathology, Michigan State University, East Lansing, MI. The antiserum was produced by hyperimmunizing rabbits with purified pili obtained from Dr. R. E. Isaacson. A 1:10 dilution of K99 antiserum in KH was used for the tests. One drop of each of the bacterial suspensions was placed on a glass slide, a drop of antiserum was added, and the two drops were mixed with a wooden applicator stick.

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[e]Baltimore Biological Laboratories, Cockeysville, MD.

The glass slide was held near a light source with a magnifying mirror and slowly rocked and tilted in a circular motion for approximately one minute. Degrees of agglutination were recorded as strong, intermediate, weak and negative. Only those E. coli 431 cultures showing strong or intermediate agglutination were used in the brush border adhesion test. Feline cultures which tested negative for agglutination were used. A control slide using KH instead of antiserum was used to check each of the bacterial suspensions for autoagglutination.

Brush Border Adhesion Test

The procedure described by Sellwood et al. (1975) was adapted for the identification of intestinal receptors for K99-positive E. coli. A 0.2 ml volume of the brush border suspension was mixed with 0.1 ml of each of the bacterial suspensions in small shell vials. The brush border-bacterial suspension was incubated at 37^{o} C with continuous gentle mixing for 30 minutes. Each E. coli culture was also incubated with 0.1 ml of KH to determine if autoagglutination was occurring. Initially, a brush border suspension with receptors for K88 pili was mixed with a K88-positive strain of E. coli (strain I248, 0157:K? Hly) as a positive control. In the latter part of the study, a known K99-positive brush border suspension was used as a positive control. Following incubation, a drop of each of the suspensions was placed on a glass slide and coverslipped. The

slide was then viewed by phase contrast microscopy[f] with a 40X objective.

Brush borders were assigned grades depending upon the numbers of bacteria adhering to the brush border fragments. The grades were as follows: Grade 1 (G1), 0-1 bacterium per fragment; Grade 2 (G2), 2-5 bacteria per fragment; Grade 3 (G3), 6-10 bacteria per fragment; and Grade 4 (G4), >10 bacteria per fragment. Brush border fragments with bacteria covering the entire microvillus surface, regardless of the number of bacteria adhering, were also considered to be Grade 4.

Electron Microscopy

To further verify piliation of the K99-positive strain of *E. coli*, a few selected cultures of *E. coli* 431 which were piliated according to the slide agglutination test were examined by transmission electron microscopy. The method used was similar to that described by Chantner (1982). The *E. coli* 431 culture was washed and suspended in distilled water. A drop of bacterial suspension was placed on Formvar carbon-coated grids[g]. After 30 seconds, the excess fluid was removed with filter paper. Next, the grids were stained for 15 seconds with 1% sodium phosphotungstate. The excess

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[f]Zeiss Photomicroscope III, Carl Zeiss, Oberkocken, West Germany.

[g]Pelco, Tustin, CA.

stain was removed, and the grids were air dried before being examined by transmission electron microscopy[h].

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[h]Zeiss Electron Microscope II, Carl Zeiss, Oberkocken, West Germany.

RESULTS

Brush Border Preparations

The brush border fragments prepared were easily discernible when viewed by phase contrast microscopy. Varying in size, the fragments were semilunar to round in shape with a distinct microvillus border. Characteristically, a halo of bright light was present around each of the brush border fragments. Cell remnants other than the microvillus border also comprised the fragment (Figure 1). Occasionally, within a brush border preparation, the attachment between adjoining epithelial cells would not be disrupted, and large fragments would be present consisting of several cells (Figure 2). Only small amounts of mucus and debris were present in most brush border preparations. However, in some preparations there was an excessive amount of mucus present. Fragments trapped in mucus or present in large numbers in close association with each other were not used for the determination of adhesion.

Bacteria

The growth conditions used to enhance piliation of the K99-positive strain of *E. coli* were successful and fairly

Figure 1. Phase contrast photomicrograph of a brush border fragment with Grade 1 adhesion. Notice the semilunar appearance of the microvillus surface (arrow) and the halo surrounding the fragment. (400X)

Figure 2. Phase contrast photomicrograph of a brush border fragment with Grade 4 adhesion. Notice at the bottom of the micrograph the large aggregate consisting of several adjoining epithelial cell fragments. (400X)

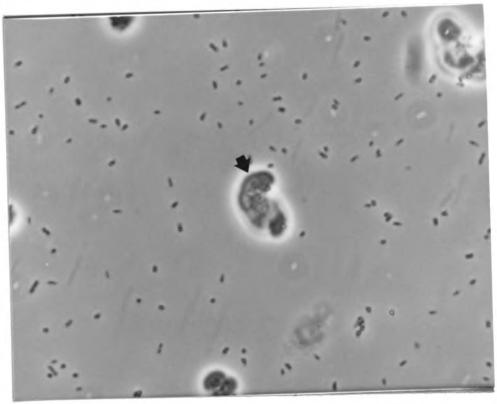

Figure 1

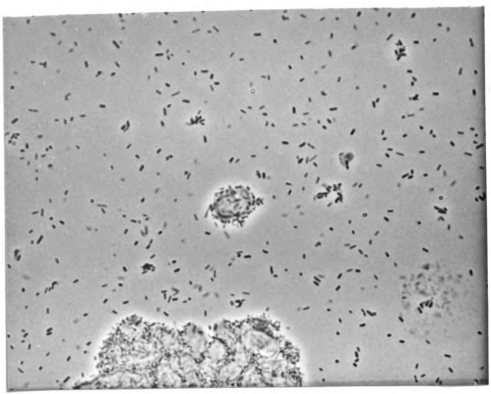

Figure 2

reliable. On the desired day of testing, piliation was confirmed by slide agglutination on 21 of 29 attempts (72%).

Pili were also demonstrated by electron microscopy on the surface of K99-positive bacteria. The pili, measuring 4.5 nm in width (Figure 3), were filamentous structures peritrichously arranged on the bacterial surface.

Adhesion

Of the 242 ileal samples examined, adhesion of K99-positive E. coli was demonstrated in 230 (95%) of the pig intestinal samples. Initially, 24 of the intestinal samples were negative for adhesion. However, adhesion was later demonstrable in 12 of the 24 samples (Table 2).

The number of K99-positive E. coli adhering to brush border fragments was less than that seen with brush border fragments with receptors for K88 pili but more than that seen with the feline negative control strain. In pigs with receptors for K99 pili, the number of bacteria adhering per brush border fragment varied from 0 to greater than 10 bacteria per brush border (Figures 1, 2, 4 and 5). Brush border fragments were present in the same preparation with no bacteria adhering to some brush border fragments and varying numbers of bacteria adhering to other fragments (Figures 6 and 7). Essentially no feline E. coli adhered to the brush border fragments. However, occasionally, one or two feline bacteria would be trapped adjacent to a brush border fragment. Hence, if only one K99-positive bacterium

Figure 3. Electron micrograph of an E. coli bacterium with K99 pili (arrows) measuring 4.5 nm in diameter. (Phosphotungstic acid stain, 55,980X)

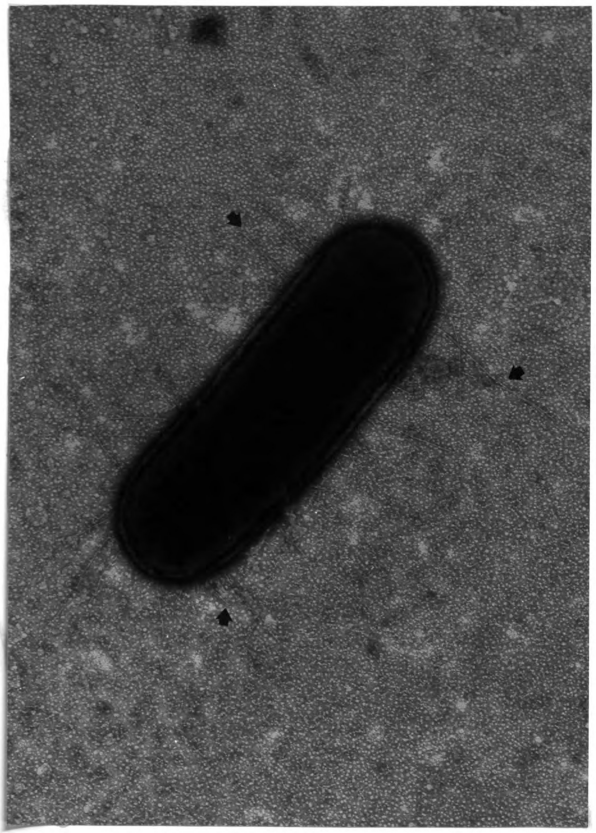

Figure 3

Table 2. Source and number of intestinal samples with or without adhesion of K99-positive *Escherichia coli*.

Slaughterhouse	Total No.	Initial Results		Final Results	
		Adhesion	No Adhesion	Adhesion	No Adhesion
1*	34	32	2	33	1
2†	60	54	6	59	1
3‡	148	132	16	138	10

*Bain's Packing Company.
†Michigan State University Meats Laboratory.
‡Milligan's Meat Packing Plant.

Figure 4. Phase contrast photomicrograph of a brush border fragment (arrow) with Grade 2 adhesion. Notice the two bacteria adhering to the fragment. (400X)

Figure 5. Phase contrast photomicrograph of a brush border fragment (arrow) with Grade 3 adhesion. Notice the 8 bacteria adhering to the fragment. Three small bacterial aggregates are also present. (400X)

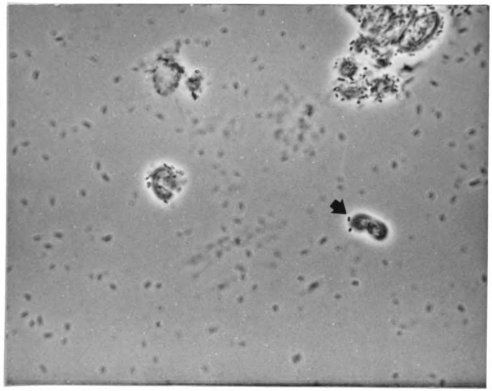

Figure 4

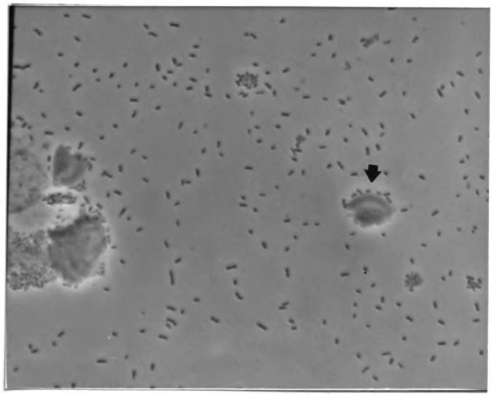

Figure 5

Figure 6. Phase contrast photomicrograph of several brush border fragments showing various grades of adhesion. All four grades of adhesion are present. The fragment in the center of the micrograph has Grade 4 adhesion. In the upper left corner, there are two fragments, one with Grade 1 adhesion and the other with Grade 2 adhesion. Three fragments are located in the lower right corner, showing Grade 1, Grade 2 and Grade 3 adhesion. An aggregate of brush border fragments is present in the upper right corner. (400X)

Figure 7. Phase contrast photomicrograph of several brush border fragments showing various grades of adhesion. The central brush border fragment has Grade 4 adhesion. The fragment on the left has Grade 3 adhesion. Notice the adhesion of bacteria along surfaces other than the microvillus surface of two of the fragments. Near the bottom of the photomicrograph is a bacterial aggregate with no brush border fragment visible beneath it (arrow). Coadherence is present on the central brush border fragment. (400X)

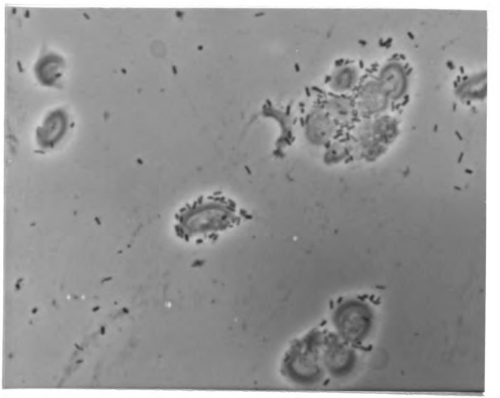

Figure 6

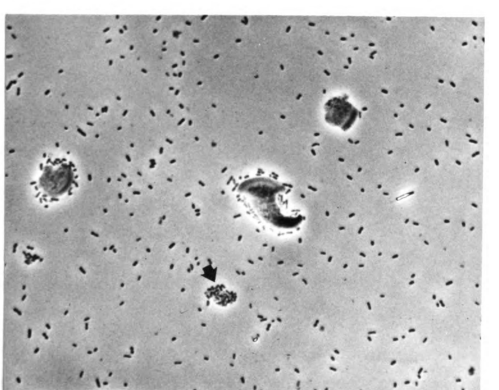

Figure 7

was noticed adhering per brush border fragment in a preparation, the sample was considered negative for receptors for K99 pili.

Adherence to the brush border fragment was not limited to the microvillus surface. Adhesion also occurred on other surfaces of the brush border fragment (Figure 7). Often, when adhesion wasn't present on the microvillus surface, K99-positive E. coli were present on other surfaces (Figure 8). Such attachment did not occur with the feline strain of E. coli.

Coadherence occurred with the K99-positive bacteria. The bacteria adhered not only to the brush border membrane, but also concurrently adhered to adjacent bacteria which in turn, were adherent to the fragment (Figure 7).

Large aggregates of bacteria were present in some preparations containing K99-positive E. coli and brush border fragments, especially in preparations refrigerated overnight (Figures 5 and 7). No brush border fragments could be identified beneath the aggregates. Sometimes small bacterial aggregates were seen in preparations with feline bacteria or preparations with K99-positive bacteria alone, but not to the extent of those aggregates seen in brush border preparations with K99-positive bacteria. While the brush border preparations were being examined by phase contrast microscopy, occasionally bacterial aggregates could be seen to detach from brush border fragments and float free in the preparation (Figures 9, 10 and 11).

Figure 8. Phase contrast photomicrograph of a brush border fragment with no K99-positive E. coli adhering to the microvillus surface but bacteria present on the other surfaces. (400X)

Figure 9. A series of three phase contrast photomicrographs (Figures 9, 10 and 11) demonstrate the detachment of a bacterial aggregate from a brush border fragment. Notice the bacterial aggregate attached to the microvillus surface of the fragment (arrow). (400X)

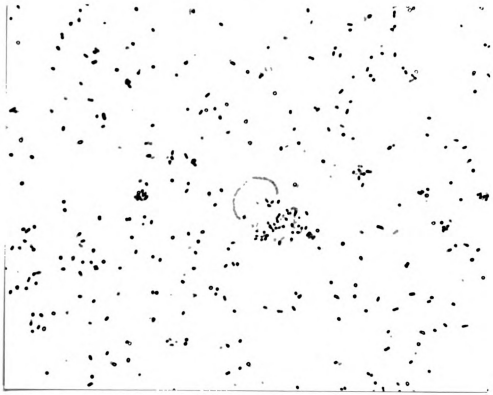

Figure 8

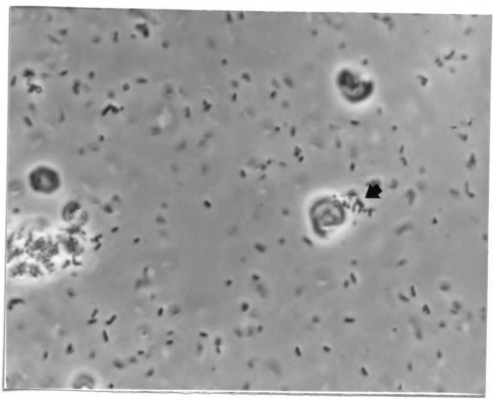

Figure 9

Figure 10. Notice that the bacterial aggregate present in Figure 9 has moved from the microvillus surface to the edge of the fragment (arrow). (400X)

Figure 11. Notice that the same bacterial aggregate present in Figures 9 and 10 has now moved to the posterior surface of the fragment (arrow). (400X)

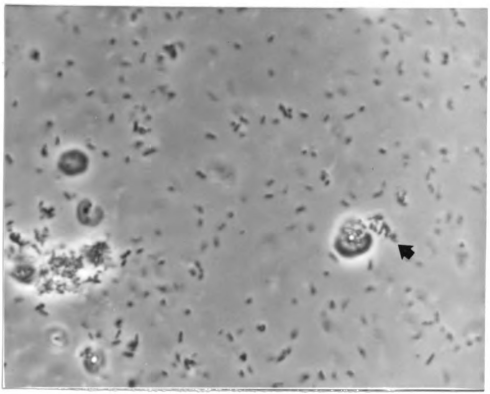

Figure 10

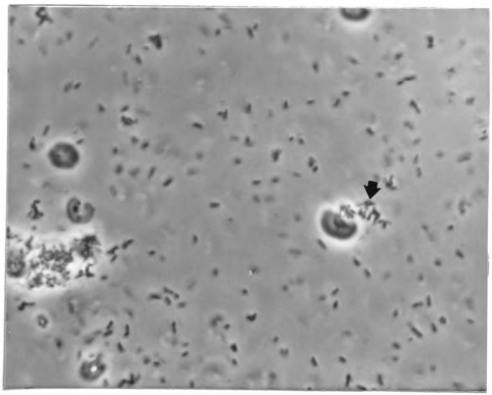

Figure 11

The distribution of the grades of adhesion of bacteria to brush border fragments from the 242 ileal samples examined is illustrated in Figure 12.

Brush border fragments stored in glycerol were well preserved. Ten different brush border preparations positive for adhesion when initially tested were reexamined for adhesion following their storage in glycerol. The adhesion results obtained from the brush borders stored in glycerol were similar to those seen in fresh preparations. The results of the brush border adhesion test are recorded in Table 3.

Using brush border preparations refrigerated for an extended period of time (> 48 hours) or stored in glycerol, the brush border adhesion test was repeated on the 24 ileal samples initially negative for adhesion. The brush border adhesion test was repeated as many as three times on the samples (Table 4).

When adhesion was demonstrated in a brush border preparation, the brush border adhesion test was not repeated on the preparation. Following the use of glycerol stored brush border preparations in the brush border adhesion test, adhesion was demonstrated in 12 of the samples which were initially negative for adhesion.

Figure 12. Distribution of the grades of adhesion of K99-positive *E. coli* to brush border fragments in 242 ileal samples.

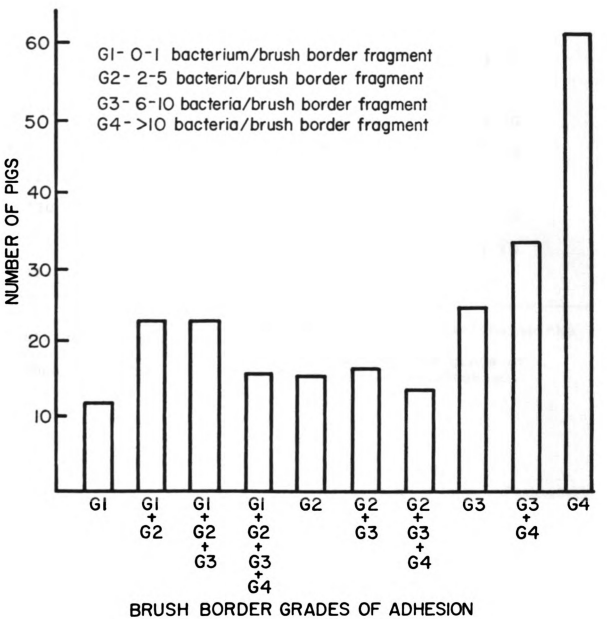
G1- 0-1 bacterium/brush border fragment
G2- 2-5 bacteria/brush border fragment
G3- 6-10 bacteria/brush border fragment
G4- >10 bacteria/brush border fragment
NUMBER OF PIGS
60
50
40
30
20
10
G1
G1 + G2
G1 + G2 + G3
G1 + G2 + G3 + G4
G2
G2 + G3
G2 + G3 + G4
G3
G3 + G4
G4
BRUSH BORDER GRADES OF ADHESION

Figure 12

Table 3. Comparison of results of brush border adhesion tests on fresh and glycerol-stored brush border fragments in 10 pigs.

Pig No.	Brush Borders		Differences in Grades of Adhesion†
	Fresh*	Glycerol	
6	G3,G4	G2,G3,G4	↓ 1G
20	G2	G2	=
48	G3,G4	G2,G3,G4	↓ 1G
68	G1,G2	G1,G2	=
98	G2,G3,G4	G1,G2,G3,G4	↓ 1G
113	G1,G2	G1,G2,G3	↑ 1G
116	G1,G2,G3	G1,G2,G3	=
130	G1,G2	G1,G2,G3	↑ 1G
131	G3	G2,G3	↓ 1G
194	G1,G2,G3	G1,G2,G3	=

*The fresh brush borders were stored overnight in the refrigerator prior to testing.

†= - no change; ↓ 1G - increased by one grade of adhesion; ↓ 1G - decreased by one grade of adhesion.

Table 4. Results of brush border adhesion retests on 24 Grade 1 intestinal samples stored in different manners.

Type of Storage*	No. of Samples	Test Results
Refrigeration	24	Large bacterial aggregates, no visible brush border fragments
Glycerol (I)	12	G1
	4	G1,G2
	2	G1,G2,G3
	2	G2,G3
	4	G3,G4
Glycerol (II)†	12	G1

*Brush border fragments were refrigerated for an extended period of time (> 48 hours) or brush border fragments were stored in glycerol.

†Using new preparations of glycerol stored brush borders, the brush border adhesion test was repeated on the 12 samples stored in glycerol which were Grade 1.

DISCUSSION

The adaptation of the brush border technique for the detection of intestinal receptors for K99 pili was successful. The primary modification consisted of growing the K99-positive strain of E. coli in liquid Minca medium with IsoVitaleX to enhance piliation. Also to insure piliation, the bacteria were transferred daily in MIB and incubated at 37° C with continuous shaking. On eight test days the bacteria were not piliated according to the slide agglutination test. Four of those times, IsoVitaleX was inadvertently excluded from the media. The reason for the lack of piliation in the other four cases is unknown. Minor variations in growth conditions, such as incubation temperature, incubation time, and rate of mechanical shaking may have influenced the state of piliation. Also, the number of broth transfers of the culture may have been a factor.

The K99-positive strain of E. coli was also demonstrated to be piliated with electron microscopy. The filamentous structures on the bacterial surface were thinner, straighter and more numerous than flagella. The pili also differed from flagella because they did not display sinous waves with a characteristic wavelength. The pili present on the K99-positive strain of E. coli were peritrichously

arranged and measured 4.5 nm in width. According to Isaacson et al. (1981), K99 pili measure < 5 nm in width.

The number of K99-positive E. coli adhering to brush borders was less than that seen with K88-positive E. coli. Isaacson et al. (1978a) observed an average of 22.5 K99-positive bacteria per epithelial cell in one day old pigs, compared to a study by Awad-Masalmeh et al. (1982) where 27.2±6.5 adherent K88-positive bacteria were present per epithelial cell. The limited and varied adhesion present in the brush border preparations from slaughter age pigs (4-6 months of age) in this study tends to concur with the results of Runnels et al. (1980) who demonstrated an age related resistance to adhesion.

The adherence of piliated E. coli to surfaces besides the microvillus surface has also been observed by other researchers. Using epithelial cells, Parry and Porter (1978) noticed the attachment of a few piliated E. coli along the basement membrane and other cellular membranes. However, most of the bacteria were tightly packed along the brush border. Sellwood (1980) called the attachment of K88-positive bacteria to components of the cell other than the microvilli nonspecific attachment. He was able to destroy the nonspecific attachment by exposing the brush borders to 1% formaldehyde. Aning and Thomlinson (1983) observed that with fresh brush borders, attachment could be seen only along the microvillus surface. However, with older brush borders, there was also attachment to the cytoplasm. Dean

and Isaacson (1982), using brush borders and epithelial cells with a 987P-positive strain of E. coli, also noted that adhesion was not restricted to the microvillus surface but occurred on the basolateral membrane as well. They considered that receptors were distributed over the entire cell surface. Some of the brush borders used by Dean and Isaacson were stored for 24 hours. In this study, the brush borders used were usually stored overnight. However, when some fresh brush border preparations were used, there was minor nonspecific adhesion. Nonspecific adhesion did not occur with the feline strain of E. coli. Receptors may be present on the entire epithelial surface and with modifications in the cell surface due to decomposition, the receptors for the pili may be exposed.

The coadherence seen in this study has also been observed by Dean and Isaacson (1982). They noticed that coadherence was not observed when brush borders were absent or when the nonpiliated mutant of the 987P strain was used.

Large bacterial aggregates, lacking identifiable brush borders, were present in this study. Dean and Isaacson (1982), using a 987P-positive strain of E. coli, also noticed large bacterial aggregates in their brush border preparations. They did not see the aggregates when fresh brush borders were used or when the nonpiliated 987P mutant strain was used. Also, when brush borders were extensively washed, bacterial aggregates were not seen. In this study, large bacterial aggregates were not seen with the feline

strain of E. coli or in preparations containing only K99-positive bacteria and no brush borders. The presence of the aggregates suggests that by some mechanism, receptors with bacteria attached to them are released from the brush border membrane. The bacteria-receptor complex then forms a clump or an aggregate. Coadherence may occur by a similar mechanism. Further evidence for the detachment of bacteria with their receptors was presented when bacterial aggregates were seen detaching from brush borders and floating free in the preparation. Recently, Dean and Isaacson (1982) identified a soluble 987P pilus receptor-containing fraction which was released from brush borders stored at 4^{o} C. Utilizing pig mucosal organ cultures, Staley and Wilson (1983) demonstrated the release of receptors for K88-positive E. coli into culture media. It appears that receptors for pili may be released in vitro from brush borders and intestinal cell membranes. The release of the receptors could lead to their isolation, purification and chemical identification.

From the results obtained with the brush border adhesion test, it appears that most, if not all, pigs have receptors for K99 pili. Hence, the results tend to confirm the assumption by Moon et al. (1979) that all pigs are congenitally susceptible to colonization by E. coli. However, in 12 of the 242 ileal samples tested, no adhesion could be demonstrated. The lack of adhesion in the intestinal samples occurred on days when adhesion was present in

other brush border preparations, as well as the control, so there is no reason to doubt that the bacteria were piliated.

Initially, adhesion could not be demonstrated in 24 of the ileal samples. The brush border adhesion test was repeated on the samples. The first time the test was repeated, brush border preparations which had been refrigerated for an extended period of time (> 48 hours) were used. No discernible brush borders were present in any of the 24 samples tested, but numerous large aggregates consisting of K99-positive bacteria were seen. As stated earlier, the presence of the aggregates suggests the possibility of receptor release with storage. Since bacterial aggregates were present in all 24 intestinal samples, it may be possible that all the samples had receptors for K99 pili.

When glycerol-stored brush border preparations were used, adhesion was demonstrable in only 12 of the 24 samples. Eight of the samples had only partial adhesion, G1, G2 and G3, perhaps indicating the presence of only a few receptors. Storage in glycerol may limit receptor release and hence, adhesion could be demonstrated in 12 samples. The 12 G1 samples which had been stored in glycerol were tested again, and no adhesion could be demonstrated. Only a very few or no receptors may have been present due to extreme age-related resistance. Storage in glycerol may not limit receptor release completely and hence, brush border

fragments with a minimal number of receptors may lose all of their receptors into solution.

Brush border preparations were routinely stored at 4° C overnight. If receptor release does occur, receptors were probably continuously lost into solution prior to testing. If brush border preparations had been tested immediately, adhesion may have been demonstrated in the 24 samples.

The retesting of 10 ileal samples initially positive before storage in glycerol, demonstrated that the results obtained from fresh and glycerol stored brush borders were similar. No differences in grades of adhesion were recorded for four samples. In four others, the adhesion results were decreased by one grade which helps to substantiate the fact that minor receptor release may occur with glycerol stored brush borders. Two samples showed an increase of one grade. Possibly, only a few fragments had the higher grade of adhesion and were not noticed in the initial test.

The possibility does exist that the 12 pigs were born without receptors for K99 pili. However, it seems unlikely because of the overwhelming number of pigs in which adhesion was demonstrated.

Results indicate that most, if not all, pigs have receptors for K99 pili. Therefore, it appears that an inheritance pattern similar to that observed for K88 receptors does not exist for K99 receptors. Additional work is needed to clarify the nature of the adhesion and how it may differ from that with K88-positive *E. coli*. Also, work is

needed on the isolation of a K99-positive receptor-containing fraction which would help characterize the receptor for the K99 pilus antigen. Dean and Isaacson (1982) have partially characterized the 987P receptor and have shown that the receptor is heat stable and that the 987P receptor-containing fraction consists of over 40 proteins.

SUMMARY

A study of 242 pigs was performed to determine if all pigs have receptors for K99-positive strains of Escherichia coli and to determine if an inheritance pattern exists for the receptors. Ileal samples, collected from pigs at three Michigan slaughterhouses, were examined using a brush border procedure modified for the identification of receptors for K99 pili. The primary modification of the brush border procedure consisted of growing the K99-positive strain of E. coli in liquid Minca medium with IsoVitaleX to enhance piliation.

Adhesion of K99-positive E. coli to brush border fragments indicated the presence of receptors for K99 pili. Of the 242 samples examined, receptors were demonstrated in 230 (95%) of the intestinal samples. From zero to greater than ten bacteria adhered per brush border fragment. The limited adhesion suggested an age-related resistance. Adhesion of bacteria to surfaces other than the microvillus surface and coadherence also was observed. Following storage of brush border preparations at 4° C, bacterial aggregates lacking identifiable brush border fragments were present in samples tested for adhesion, suggesting the release of K99 receptors from the brush border membrane.

In this study, the brush border procedure was successfully modified for the identification of K99 receptors. The results obtained from the brush border adhesion test indicate that most, if not all, pigs have receptors for K99 pili. Therefore, it appears that an inheritance pattern similar to that observed for K88 receptors does not exist for K99 receptors.

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