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By

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ABSTRACT

PILIATED ESCHERICHIA COLI AND
PORCINE ENTERIC DISEASE IN MICHIGAN

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A study of 125 neonatal pigs was performed to determine the prevalence of pilus antigens of enterotoxigenic Escherichia coli in Michigan swine herds. Live, diarrheic pigs under 2 weeks of age submitted for study were euthanatized and frozen sections of ileum were subjected to an indirect fluorescent antibody technique to detect the 3 known pilus antigens of E. coli known to affect young swine (K88, K99, and 987P). Ten centimeter ileal sections were used to determine numbers of lactose-fermenting bacteria.

Of 52 pigs in which pili of E. coli were demonstrated, 14 had K88 (27%), 23 had K99 (42%), 13 had 987P (25%) and 2 had K88 and K99 simultaneously (4%). Numbers of lactose-fermenting bacteria were significantly higher ($p<0.05$) in pigs with piliated E. coli than in pigs without piliated E. coli.

This study suggests that piliated E. coli are a significant cause of enteric disease in Michigan neonatal swine, and that the K99 pilus antigen is most frequently encountered.

DEDICATION

To my family

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INTRODUCTION

Neonatal diarrhea in pigs is one of the most common causes of morbidity and mortality in swine herds. In Michigan, enteric diseases of young swine are considered to be a major obstacle toward increased profitability of swine production. Enteropathogenic Escherichia coli are responsible for a substantial portion of the economic losses due to enteric disease.

Diarrheal disease caused by enteropathogenic E. coli (enteric colibacillosis) has long been recognized as a problem in Michigan, yet only recently have definitive diagnostic procedures been developed. The demonstration of certain adherence-promoting, hair-like organelles (called pili or pilus antigens) on the bacterial surface of E. coli and the positive correlation of these structures with enterotoxigenicity has contributed to a better elucidation of the pathogenesis of enteric colibacillosis. Subsequently, vaccines which hold the promise for a reduction of the incidence and severity of this disease in swine have recently been developed. A determination of the prevalence of piliated E. coli in Michigan neonatal swine will permit such vaccines to be of maximal value.

The objectives of this study were:

1) To evaluate the usefulness of an indirect fluorescent antibody technique to identify K88, K99, and 987P pilus antigens of Escherichia coli in the ileum of neonatal pigs submitted with a history of enteric disease.

2) To compare, by quantitative methods, the number of lactose-fermenting bacteria in a 10-cm length of ileum in pigs with and without enteric colibacillosis.

3) To determine the prevalence of E. coli with each of the 3 pilus antigens in neonates in Michigan swine herds having enteric disease.

LITERATURE REVIEW

Pathogenic Escherichia coli have been incriminated as a cause of porcine enteric disease since 1899 (Jensen, 1948). Several reviews of this disease in pigs (Barnum et al., 1967; Nielsen et al., 1968; Kohler, 1972) and other species (Tennant, 1971) have been published. There is a general consensus among investigators that diarrheal disease caused by pathogenic E. coli is one of the most economically important diseases in pigs and other species (Sojka, 1965).

Not all serotypes of Escherichia coli cause enteric disease, as some are a component of the normal flora of the intestinal tract of man and animals. The organism is a gram-negative, non-sporeforming rod of the family Enterobacteriaceae and is 2-3 μm in length and 0.6 μm in width. The bacteria may be motile or nonmotile and are classified as lactose fermenters. Illness caused by pathogenic strains of E. coli is widespread due to the ubiquitous nature of pathogenic strains of the organism. The term enteric colibacillosis has been used to describe the intestinal disease caused by pathogenic E. coli (Wilson, 1981).

Clinical Signs

Enteric colibacillosis in neonatal pigs is most commonly seen as an acute enteritis. The disease has been referred to by several names, including white scours, baby pig scours, coliform enteritis, diarrhea neonatorum, and neonatal colibacillary disease (Leman, 1970). The pigs affected by the disease are often less than 4 days of age. However, the disease may strike at any time before weaning. Some pigs become ill as soon as 12 hours after birth (Dunne, 1975). Post-weaning enteric colibacillosis does occur, but death losses are fewer in this age group.

Neonatal enteric colibacillosis in pigs is characterized by variable morbidity. Not all litters will be affected, nor will all pigs in an affected litter show signs of illness (Barnum et al., 1967). Mortality is also variable depending upon the age of the animal. Dunne and Bennett (1970) reported a mortality rate of approximately 70% in pigs 3 days of age or less, but mortality rarely reaches 100% (Leman, 1970).

The clinical signs of enteric colibacillosis in young pigs include the passage of whitish-yellow, watery feces. This is often followed by dehydration. Vomition is usually absent but may be present in severe outbreaks (Wilson, 1981). Anorexia may be apparent. However, many suckling pigs having diarrhea continue to nurse. The hair coat may appear roughened, and the perineum may become irritated due to the continual presence of fluid feces (Dunne, 1975).

Lesions

There are no pathognomonic lesions, either gross or microscopic, in pigs with enteric colibacillosis. The gross lesions of this disease may include mild to severe inflammatory reactions. Occasionally, no inflammatory changes are present. The stomach is sometimes partially filled with curdled milk but lacks gross lesions. The intestines are usually distended and contain visible amounts of yellowish to grayish contents, often with excessive mucus. Hyperemia and edema may be seen in the small and large intestines, and petechial and ecchymotic hemorrhages may be present throughout the gastrointestinal tract. Villous atrophy, usually associated with coronavirus infection, is occasionally seen with colibacillosis (Kenworthy and Allen, 1966; Moon et al, 1970).

Microscopically, the changes seen in colibacillosis in conventionally-reared pigs are variable. Smith and Jones (1963) reported no inflammatory changes in the intestinal tract in pigs with enteric colibacillosis, while Dunne and Bennett (1970) noted enlargement of goblet cells and vacuolation of absorptive cells. Edema in the lamina propria and neutrophilic infiltration into villi have been noted (Christie and Waxler, 1972). A layer of adherent rod-shaped bacteria is often seen contiguous to the tips and lateral surfaces of small intestinal villi (Wilson, 1981).

Pathogenesis

Many recent advances have contributed to our understanding of enteric colibacillosis as it occurs in swine. In the past, scientists had great difficulty explaining why some serotypes of E. coli caused disease while others did not. However, the identification and characterization of certain virulence factors of some E. coli have been a beginning in the quest for a more complete understanding of the pathogenesis of this disease.

Enterotoxins

One virulence factor of enteropathogenic E. coli is its ability to produce enterotoxins. The production of enterotoxins was demonstrated by Skerman et al. (1972) to be mediated by plasmids. These enterotoxins are generally classified according to their thermal stability, and this classification is generally in accord with different cellular activities of these toxin types (Evans et al., 1973a). Smith and Halls (1967) demonstrated that certain E. coli produced a heat-stable (ST) enterotoxin while Gyles and Barnum (1969) demonstrated a heat-labile enterotoxin (LT). The heat-stable toxin was found to resist temperatures of 100^{o} C for 15 minutes; heat-labile toxin became inactivated at 60^{o} C for 15 minutes. Enterotoxigenic E. coli may produce either LT, ST, or both classes of enterotoxins (Gyles, 1970).

The antigenic properties of LT and ST differ. The LT has been shown to have antigenic behavior (Finkelstein et al., 1975), and Kohler (1978) has demonstrated that antibodies made against LT were helpful in preventing disease. The ST is nonantigenic (Smith and Halls, 1967). However, pigs develop resistance with age to some, but not all, strains of enterotoxigenic E. coli that produce only heat-stable enterotoxin (Moon and Whipp, 1970). Whipp et al. (1980) concluded that these strains may produce an inhibitor which interferes with the cellular secretory response of older pigs to ST.

The structure of LT has been investigated. It is comprised of a single polypeptide chain with a molecular weight of 100,000 Daltons (Evans et al., 1976) and is related to the cholera toxin in its antigenicity (Gyles, 1974; Klipstein and Engert, 1977). The effect of LT in the intestinal epithelial cell is to increase levels of membrane-bound adenylate cyclase with a subsequent increase in the levels of intracellular cyclic adenosine 3',5'-monophosphate (cAMP). This causes a net fluid and electrolyte secretion by the epithelial cells of the small intestine, and the disease produced is therefore defined mechanistically as a secretory diarrhea (Moon, 1978a). Other nonintestinal cells, such as HeLa cell monolayers used in cell culture, have also been found to be sensitive to LT (Keusch and Donta, 1975). The mechanism of cAMP-mediated hypersecretion probably accounts for the diarrhea in other

diseases such as salmonellosis and human cholera, both of which involve toxin production by bacteria (Moon, 1978a). Recently, Newsome et al. (1978) suggested that LT-induced intestinal secretion may be mediated by changes in the cyclic adenosine 3',5'-monophosphate/cyclic guanosine 3',5'-monophosphate (cGMP) ratio.

The ST toxin of E. coli has not been fully characterized, and occasional reports indicate that different types of ST exist (Guerrant et al., 1975; Steiner et al., 1972). Newsome et al. (1978) reported on 2 subtypes of ST from E. coli strain P16 and designated them as ST_A and ST_B. The ST_A component was soluble in methanol, active in the 1-3 day old piglet, but inactive in the weaned pig. ST_B was insoluble in methanol, inactive in the 1-3 day old piglet, but active in the weaned pig. Available reports indicate that ST may induce diarrhea by a mechanism other than via adenylate cyclase (Gianella, 1977; Hamilton et al., 1978). Hughes et al. (1978) and Newsome et al. (1978) concluded that either an increase in cGMP concentration or a decrease in the ratio of cAMP/cGMP may be the mechanism of action of ST_A, but little is known about the mechanism for the effects of ST_B.

Kapitany et al. (1979) isolated an ST from porcine enterotoxigenic E. coli and designated it as ST-1261. This toxin had distinct similarities in chemical composition to the ST-431 isolated from a different strain of porcine enterotoxigenic E. coli by Alderate and Robertson (1978).

However, it was difficult to make comparisons of these enterotoxins to the ST_A and ST_B previously described. The purification of heat-stable enterotoxins of E. coli has apparently been complicated by the complexity of the various growth media employed for enterotoxin production (Alderate and Robertson, 1978). Furthermore, the apparent heterogeneity of ST may be based on the sensitivity of the various test systems used for its purification (Burgess et al., 1978).

There are several methods available to detect enterotoxins of E. coli. An in vivo assay for heat-stable and heat-labile enterotoxins is the gut loop test in which ligated segments of small intestine of an anesthetized pig are injected intraluminally with a test inoculum. Distention of the loop after 24 hours indicates a positive reaction for enterotoxin (Smith and Halls, 1968). This test is most sensitive and reproducible when isolates are tested in the same species from which they were originally obtained.

More recent methods for the detection of E. coli enterotoxins have been developed. Tissue cultures of Y-1 adrenal cells (Donta et al., 1974) or Chinese hamster ovary (CHO) cells (Guerrant et al., 1974) are used to detect LT. ST does not react in these tissue culture techniques. An in vivo technique for detection of LT is the rabbit skin test. This test is based on the effects of LT on vascular permeability (Evans et al., 1973b).

Several immunologic methods are used to detect LT. A lysis inhibition test (Evans and Evans, 1977), a radioimmunoassay technique (Greenberg et al., 1977), and an enzyme-linked immunosorbent assay (ELISA) have been described (Yolken et al., 1977). The lack of antigenic properties of ST make it inappropriate for detection by immunologic methods.

Heat-stable enterotoxin may be detected by the infant mouse test, in which a test inoculum is injected into the stomach of the young mouse, and the subsequent fluid secretion into the intestines is measured. Of the 2 kinds of ST provisionally recognized, only ST_A is detected by the infant mouse test; the ligated gut loop technique in the pig is used to detect ST_B (Newsome et al., 1978).

Adhesion

A second virulence attribute of enterotoxigenic E. coli is the ability to adhere to intestinal epithelial cell surfaces. It is the property of adherence to those cell surfaces that permits the microorganism to resist the peristaltic movement of ingesta and therefore avoid being washed out of the small intestine. In general, those bacterial organelles that promote adhesion to cell surfaces are defined as adhesins. These adhesins allow the E. coli to reproduce in great numbers, a process known as colonization (Nagy et al., 1976). The antigenic structures responsible for adherence and colonization of enterotoxigenic E. coli in many species of animals have been

identified by several researchers (Burrows et al., 1976; Nagy et al., 1977; Ørskov and Ørskov, 1966; Ørskov et al., 1975; Smith and Linggood, 1971). In each case, the adhesins were found to be hair-like projections, designated as pili or fimbriae, on the surface of the bacterium. The presence of pili is associated with ability of the organism to produce enterotoxin in most field isolates of enterotoxigenic E. coli (Isaacson, 1981).

Brinton (1959) was the first to use the term pili (Latin for hairs) to describe the nonflagellar structures projecting from the bacterial surface. Duguid (1955) used the term fimbriae (Latin for fringe) to identify the same appendages. This class of pili is not involved in the transfer of DNA between bacteria, a function of conjugal or sex pili. Other classes of pili are known as type I somatic, or "common", pili. These have no role in conjugation and are coded for by chromosomal genes. Common pili are hollow fibers 7 nm in diameter and are 500-2000 nm in length. A given bacterial cell may have 50-400 per bacterium (Brinton, 1959, 1967). Common pili enhance upon bacteria many behaviors lacking in the nonpiliated phase. These behaviors include surface translocation, enhanced growth in marginal oxygen concentrations, tight colonial association of bacterial growth, the ability to agglutinate guinea pig erythrocytes, and the ability to adhere to cell surfaces (Brinton, 1978). The hemagglutinating and adherence properties are eliminated by the monosaccharide

D-mannose. This observation has lead to the term "mannose-sensitive hemagglutination and adhesion" (MSHA) and is a characteristic of adhesion mediated by type I somatic pili (Duguid et al., 1955). Type I piliated E. coli may be distinguished from nonpiliated organisms by colonial morphology (Salif and Gotschlich, 1977a, 1977b). However, the role of common pili in the colonization of enterotoxigenic E. coli has not been proven (Isaacson, 1980).

The K88 Pilus. Ørskov and Ørskov (1961) isolated E. coli from piglets with diarrhea and described a new pilus antigen designated as K88. It was designated as a capsular (K) antigen due to its serologic behavior during serotyping procedures. However, it was later recognized that the pili were protein antigens rather than polysaccharides (as reviewed by Moon, 1978b). The protein has a high molecular weight which can be separated into apparently identical subunits of approximately 25,000 Daltons each (Mooi and DeGraaf, 1979).

Ørskov et al. (1964) demonstrated that K88 existed in two distinguishable forms, designated as K88ab and K88ac, but Stirm et al. (1967a) were the first to isolate and partially purify K88ab. Recently, a new variant, provisionally designated as K88ad, has been described by Guinée and Jansen (1979). These subtypes of the K88 antigen are serologic variants which have minor differences in their amino acid compositions. Each variant of K88 can be detected by immunodiffusion and immunoelectrophoretic

techniques (Mooi and DeGraaf, 1979).

Stirm et al. (1967b) found that K88 was morphologically distinguishable from type I somatic pili in electron micrographs. The K88 antigen appeared as fine fibers on the bacterial surface. These pili were 100-1500 nm long and 7-11 nm wide. Ørskov and Ørskov (1966) found that K88 pili were plasmid-mediated and that nutrient requirements in vitro for K88 expression were fastidious. The K88 antigen was expressed after growth on solid medium at 37° C but not at 18° C. Stirm et al. (1967b) demonstrated that enterotoxigenic E. coli bearing the K88 pilus had the ability to agglutinate guinea pig red blood cells in the absence and presence of D-mannose.

Smith and Linggood (1971) demonstrated that K88 was a virulence factor of K88-positive strains of E. coli that cause diarrhea in neonatal pigs. These strains manifested adhesive behavior to intestinal epithelial cells, permitting attachment and colonization in the small intestine. It was further demonstrated that K88-negative mutants of K88-positive strains were nonenteropathogenic because they could not attach to enterocytes and therefore not colonize the intestine, despite being comparable in their abilities to produce enterotoxin.

Jones and Rutter (1972) demonstrated that K88 antisera inhibited the in vivo attachment of the K88 pilus and concluded that antibodies directed against K88 could prevent its adherence to porcine intestinal epithelium. This

discovery has been an impetus for the development of veterinary vaccines against enteric disease caused by piliated E. coli.

Smith and Linggood (1971) also found that E. coli bearing K88 did not attach to the intestinal epithelium of all nonvaccinated piglets. Rutter et al. (1975) described 2 different phenotypes in pigs based on the ability of K88 positive E. coli to adhere. The "adhesive" phenotype was presumed to possess a receptor for the K88 pilus that promoted preferential attachment to it. These pigs were more susceptible to colibacillosis from E. coli bearing this pilus than the "nonadhesive" phenotypes which lacked the receptor. Gibbons et al. (1977) demonstrated that the receptor for K88 is inherited in a simple Mendelian fashion, with 1 locus and 2 alleles, and the dominant allele is expressed as the receptor for K88 on the microvillous surface. The homozygous recessive pig lacks this receptor. Sellwood et al. (1975) have described an in vitro assay in which the interaction between K88-bearing E. coli and the intestinal brush borders was directly observed. Sellwood (1980) has recently demonstrated a technique which allows measurements of the K88/receptor interaction. Attempts to further define the chemical properties of the receptor utilizing the ability of soluble K88 pilus antigen to agglutinate guinea pig erythrocytes have been made. Gibbons et al. (1975) concluded that hemagglutination can be inhibited by some glycoprotein moieties, especially the galactosyl

residue. However, much remains to be learned about the chemistry of the receptor for K88.

Recently, Bijlsma et al. (1982) described a further subdivision within the group of "adhesive" animals by demonstrating different provisional phenotypes in pigs distinguishable with the 3 variants of the K88 antigen, using the brush border technique of Sellwood et al. (1975). Pigs of phenotype A were susceptible to adherence of all three K88 variants; pigs of phenotype B were susceptible to K88ab and K88ac; pigs of phenotype C were susceptible to K88ab and K88ad; pigs of phenotype D were exclusively susceptible to K88ad; and pigs of phenotype E were resistant to adhesion by any of the K88 variants.

It is of interest that the K88 antigen has been shown to be a virulence factor in only 1 species, the pig (Moon, 1978b).

The K99 Pilus. Smith and Linggood (1972) described another antigen of E. coli and designated it as K_{CO}. They demonstrated that it was a plasmid-mediated antigen and that when the plasmid was removed from enterotoxigenic lamb strains the organism was not diarrheogenic. These researchers further demonstrated that K_{CO}-positive strains colonized the small intestine in high numbers and speculated that K_{CO} in lamb and calf enterotoxigenic E. coli strains may serve a similar function as K88. K_{CO} later became officially designated as K99 (Ørskov et al., 1975) and was speculated to have a structure resembling pili.

The K99 pilus antigen was found to be similar to K88 in many respects. Burrows et al. (1976) demonstrated that K99, like K88, was expressed after cultivation on solid agar at 37° C but not at 18° C and that E. coli bearing K99 caused mannose-resistant agglutination of ovine erythrocytes. Ørskov et al. (1975) confirmed that genes for K99, like K88, were found in plasmids. Both K88 and K99 have a similar appearance on electron micrographs (Isaacson, 1977).

The K99 pili were purified by Isaacson (1977). It is characterized by rod-like structures that averaged 8.4 nm in diameter and 130 nm in length. The antigen was composed of 2 subunits that were mainly protein with a minor lipid component. The major subunit had a molecular weight of 22,500 Daltons, and the minor subunit had a molecular weight of 29,500 Daltons. Isaacson (1977) confirmed that K99 had the structure of pili.

The nutrient requirements in vitro that promote expression of K99 have been explored. Guinée et al. (1976) reported that K99 could be more easily demonstrated when strains were cultured on a buffered medium containing a minimal amount of casein (Minca medium). The supplementation of this medium by 1% IsoVitaleX[a] gave improved results (Guinée et al., 1977). Isaacson et al. (1978b) further improved this method by growing K99 strains on Minca-IsoVitaleX agar. Cultures that tested negatively

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[a]Baltimore Biological Laboratory, Baltimore, MD.

for K99 by slide agglutination were then passaged daily for 4 days in trypticase soy broth with vigorous shaking. Strains were then regrown on Minca-IsoVitaleX agar.

Unlike the K88 antigen, K99 has been associated with enterotoxigenic strains of E. coli in lambs and calves (Ørskov et al., 1975). Moon et al. (1977) detected K99 from enterotoxigenic E. coli isolated from piglets and demonstrated that calf strains of E. coli bearing K99 were enteropathogenic for pigs.

Little is known about the intestinal receptor for K99 in the pig, lamb, or calf. Data suggest, however, that pilus receptors exist in the small intestine that recognize only a single pilus type (Isaacson et al., 1978c). Moon et al. (1979) have speculated that all neonatal pigs have intestinal receptors for K99 indicating that there is no inheritable resistance in pigs to infection with K99-bearing E. coli. However, this has not been rigorously established.

The 987P Pilus. A third pilus antigen of E. coli has been described by Isaacson et al. (1978c). Many enterotoxigenic E. coli strains isolated from piglets with diarrhea lacked K88 and K99, yet were clearly enteropathogenic. These strains adhered to porcine enterocytes in vivo and in vitro and induced diarrhea when given orally to piglets. One such strain, E. coli 987, has been found to adhere by a class of pili that are different from both K88 and K99. Escherichia coli 987 pili had similar dimensions to common pili in electron micrographs, yet they differed

biochemically and antigenically. Moreover, unlike common pili, E. coli 987 pili did not hemagglutinate guinea pig erythrocytes (Isaacson et al., 1978c; Nagy et al., 1977). The 987P pili have been detected in calf, lamb, and pig strains of E. coli, but they are a virulence factor only in the pig (as reviewed by Moon, 1978b). It is not known whether the genes that code for 987P pili are located in plasmid or chromosomal nucleoproteins (Moon et al., 1979).

Recently, 2 enterotoxigenic strains of E. coli from different sources were both found to express 987P and K88 pilus antigens at different times. This indicates that some E. coli occur naturally with the potential to produce more than 1 of the pili that promote colonization (Schneider and To, 1982). It would be helpful to determine if strains which produce more than 1 pilus antigen produce them both in vivo and if they both promote colonization of such strains.

Little is known about the nature of the intestinal receptor for the pilus of E. coli 987 in the pig. Dean and Isaacson (1982) used an in vitro laboratory model to identify and isolate a 987P pilus-specific receptor-containing fraction from small intestinal epithelium and brush borders isolated from adult female rabbits. Interestingly, the receptor-containing fraction was not found in neonatal rabbits. Moon et al. (1979) have suggested that, as in the case of receptors for K99, all neonatal pigs have intestinal receptors for 987P.

Unidentified Adhesins. Recently, Amad-Masalmeh et al. (1982) described 3 strains of enterotoxigenic E. coli that adhered, colonized, and caused diarrhea in pig intestine but which did not produce K88, K99, or 987P antigens. Such strains were designated as $3P^-$ strains. These workers concluded that $3P^-$ enterotoxigenic E. coli produce previously uncharacterized adhesins and demonstrated that certain strains did produce pili but that these pili apparently did not promote adherence or colonization. Apparently, the adherence of $3P^-$ strains to enterocytes is mediated by adhesins that are not of a pilus nature.

Morris et al. (1982) documented a new filamentous antigen, provisionally designated as F41, which was produced by a strain of E. coli infective for calves that was a K99-negative mutant of the K99 reference strain B41. Adhesiveness was demonstrated both in vivo and in vitro, and the antigen was shown to have mannose-resistant hemagglutination properties. While these features point to the possibility of F41 being a new pilus antigen of enterotoxigenic E. coli, experiments are necessary to clarify the role of F41 in the pathogenesis of diarrheal disease.

Moon (1978b) has suggested evidence for previously uncharacterized pilus antigens. These strains have the ability to colonize intestines of neonatal pigs and yet lack K88, K99, or 987P. Such E. coli may yield new pilus antigens. However, Francis (1982) has indicated that the

vast majority of piliated E. coli found in a prevalence study in north central United States bore K88, K99, or 987P pili.

Diagnostic Techniques for Pilus Identification

The pilus antigens of E. coli may be detected by several techniques. An enzyme-linked immunosorbent assay (ELISA) has been used to detect K99 in calf feces and K99 antibody in serum and colostrum of calves (Ellens et al., 1978). These results indicate that K99-positive E. coli infections can be diagnosed serologically. Mills et al. (1982) recently described an ELISA for the detection of K88 pili.

A slide agglutination test for detection of K99 has been reported by Guinée et al. (1976). When Minca or Minca-IsoVitaleX medium was employed, the K99 antigen was more readily detected (Guinée et al., 1977). A modification of this technique utilizing trypticase soy broth gave improved results (Isaacson et al., 1978b).

Probably the most suitable technique for the demonstration of pilus antigens is the indirect fluorescent antibody technique. Several workers (Moon, 1978b; Isaacson et al., 1978a, 1978b; Moon et al., 1978, 1980) recommended immunofluorescent staining of bacteria in frozen ileal sections using specific K88, K99, or 987P antisera for a sensitive and simple technique for the detection of these pilus antigens.

Vaccination with Pili

Because enteric colibacillosis in piglets usually occurs during the neonatal period, the disease is controlled by immunization of pregnant sows to stimulate colostral antibodies which protect piglets that suckle such dams (Rutter et al., 1976). Controlled experiments have been done using purified pili as parenteral vaccines with subsequent challenge of suckling piglets with strains of enterotoxigenic E. coli bearing a homologous pilus antigen. Protection correlated with the presence of anti-pilus antibodies in the colostrum and milk of immunized sows (Nagy et al., 1978; Rutter et al., 1976). In other experiments, piglets born to control and immunized dams were then challenged with a strain of enterotoxigenic E. coli bearing the homologous pilus antigen, but different cell wall (O) and flagellar (H) antigens from those of the strain from which the vaccine was prepared. The results again indicated that vaccine effectiveness was determined by the presence of anti-pilus antibodies (Morgan et al., 1978). These studies concluded that up to 80 or 90% of E. coli diarrhea in pigs can be prevented by use of a trivalent pilus vaccine containing purified K88, K99, and 987P pili.

MATERIALS AND METHODS

Immunofluorescence Procedures

Control Animals

Gnotobiotic pigs were obtained from a Hampshire sow using a technique described by Waxler, et al. (1966). Epidural anesthesia was induced by injection of 25 ml of 2.5% procaine hydrochloride[b] given at the lumbosacral articulation, followed by 5 ml of tranquilizer given intramuscularly[c]. The sow was positioned in right lateral recumbency. The left flank was surgically prepared and sprayed with a sterile surgical adhesive[d], and the plastic isolator was positioned over the area.

The surgeon cut through the vinyl floor of the isolator unit and the sow's skin with a cautery scalpel unit. The remaining layers of the abdominal wall were incised with scissors. Each pig was removed from the uterus by making a separate incision in the uterine wall. Umbilical clamps[e] were placed on the umbilical cord of each pig prior to severing the cord. The pigs were then aseptically

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[b]Epidural, Haver-Lockhart, Kansas City, MO.
[c]Sparine, Wyeth Laboratories, Philadelphia, PA.
[d]Vi-Drape, Parke-Davis, Detroit, MI.
[e]"Double Grip" disposable cord-clamp, Hollister, Inc., Chicago, IL.

transferred into a plastic holding isolator. The litter consisted of 8 live pigs, 7 of which were used in this study. The 7 pigs were allocated to 4 separate isolators, with 2 pigs in 3 isolators and 1 pig in a fourth isolator. Equipment for feeding and for microbiologic determination was contained in each isolator. A sufficient quantity of a commercial sterilized liquid diet[f] was aseptically transferred into each isolator, and each pig was given 3 to 4 oz. of liquid diet 3 times a day. Each pig was maintained in an individual cage in sterile plastic rearing isolators at a room temperature of 30-32°C.

Bacteriologic Monitoring. Swabs were taken from each isolator prior to the pigs' exposure to the test inoculum. Specimens of rectal contents, waste material from the cages, and contents of feeding trays were taken. Material was streaked on blood agar[g] plates and inoculated into thioglycollate broth[g]. The media were incubated both aerobically and anaerobically at 25°, 37°, and 55°C.

Control Inocula. Cultures of 3 strains of E. coli of known pilus type growing on trypticase soy agar were obtained from Dr. R. E. Isaacson, School of Public Health, University of Michigan, Ann Arbor (Table 1). The strains were transferred to trypticase soy broth and incubated aerobically overnight at 37°C.

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[f]SPF-Lac, Borden Inc., Norfolk, VA.
[g]Difco Laboratories, Detroit, MI.

Table 1 - *Escherichia coli* strains and pilus antigens.

Strain	Pilus Antigen
I248	K88
431	K99
987	987P

Three milliliters of the broth culture of each strain were placed in individual 5 ml sterile glass ampules and heat sealed. An ampule containing only trypticase soy broth with no bacteria was also prepared in this manner. The ampules were aseptically transferred into the appropriate isolators just prior to exposure of the pigs. At 7 days of age, each pig was orally inoculated with 1 ml trypticase soy broth culture (Table 2).

Table 2 - Number and distribution of pigs receiving 1 ml of trypticase soy broth (TSB) cultures.

Isolator Number	Number of Pigs	Inoculum
1	1	TSB only
2	2	Strain 987 in TSB
3	2	Strain I248 in TSB
4	2	Strain 431 in TSB

Quantitation of E. coli from Control Inocula. One milliliter of each inoculum given to the gnotobiotic pigs was serially diluted in 9 ml of phosphate-buffered saline. Eleven 10-fold serial dilutions were made. One milliliter of each dilution from 10^{-4} to 10^{-8} was cultured using MacConkey agar and a standard pour plate technique. All plates were incubated overnight at 37°C. Because dilutions at 10^{-1}, 10^{-2}, and 10^{-3} would probably yield growth that was too numerous to count, cultures of those dilutions were not made. Dilutions in which 30 to 300 colonies could be counted were used in the calculation of the number of E. coli.

Determination of Clinical Signs. Pigs were observed 3 times daily for clinical signs of anorexia, dehydration, diarrhea, and vomiting throughout the duration of the experiment.

Necropsy Procedures. The pigs were euthanatized 24 hours after exposure to the inocula by administering 2.0 ml of sodium pentobarbitol[h] into the anterior vena cava. Pigs were then necropsied in dorsal recumbancy by incising and caudally reflecting the sternum and ventrum. Sections of duodenum, jejunum, and ileum were frozen in OCT[i] mounting medium, and other sections of these same tissues were fixed in 10% neutral buffered formalin. Formalin-fixed intestinal sections were stained with hematoxylin and eosin as well as

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[h]Haver-Lockhart Laboratories, Kansas City, MO.

[i]Tissue-Tek II O.C.T. Compound, Lab-Tek Products Division, Miles Laboratories, Inc., Naperville, IL.

Giemsa stains. Sections of kidney, liver, lung, lymph node and spleen were also formalin-fixed and stained with hematoxylin and eosin.

Preparation of Antibodies

The monospecific anti-pilus antibodies were prepared in this laboratory prior to the beginning of this study. The method of preparation is summarized in the Appendix.

Preparation of Tissues for Indirect Fluorescent Antibody Procedure

The gnotobiotic pigs served as controls to determine the monospecificity of each anti-pilus antibody preparation. Sections of ileum 5 cm in length were taken adjacent to the ileocecal junction. Sections were ligated at 1 end with suture material, and the embedding medium was introduced into the lumen of the intestine until it was mildly distended. The open end was then ligated. The tissue was placed in a container with dry ice to harden the embedding medium. Care was taken to not allow direct contact of the tissue with the dry ice. Sections of embedded ileum were then cut into cross sections 3-5 mm in length. Selected sections were placed on a cork disc 2.2 cm in diameter and 0.3 cm in thickness. Additional embedding medium was added to cover the cross sections. Tissues were then placed into small labelled plastic bags, sealed, and stored at -70^{o}C.

When sections of frozen, embedded tissue were utilized for the indirect fluorescent antibody procedure, they were attached onto metal cylinders. Six-micron thick cross

sections were cut using a cryostat[j]. Sections were mounted on glass slides, heat-fixed at 37°C in a moist chamber, and fixed in acetone for 15 minutes at room temperature. Slides were allowed to dry, and individual tissue sections were overlaid with the anti-pilus antibodies made in the rabbit. Eight ileal sections from each animal were used. Two sections had anti-K88 antibody placed over them; two sections had anti-K99 antibody placed over them; two sections had anti-987P antibody placed over them. The remaining 2 sections had normal rabbit serum placed over them. All antisera were used at a dilution of 1:100 in phosphate buffered saline (PBS) at pH 7.2. Slides were incubated in a moist chamber for 30 minutes at 37°C followed by 2 rinses in PBS at pH 7.2. The second antibody was a commercially available anti-rabbit IgG fluorescein conjugate made in the goat[k,l]. It was used at a dilution of 1:16 in PBS at pH 7.2. Slides were incubated in a moist chamber at 37°C as before. Two PBS rinses were made. The slides were dried and coverslipped with a 1:9 mixture of PBS and glycerol.

The mounted specimens were examined on a Zeiss Photomicroscope III[m] with immunofluorescence capacity. All tissues were examined the same day they were processed. A

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[j]South London Electrical Equipment Co., Ltd., London, England.
[k]Miles-Yeda Ltd., Kiryat Weizman, Rehovot, Israel.
[l]Antibodies Incorporated, Davis, CA.
[m]Carl Zeiss, Oberkocken, West Germany.

specimen was considered positive for pilus antigens if specific, apple-green-colored fluorescence was noted on the tips and lateral surfaces of villi.

Procurement of Field Specimens

The animals utilized for this study were pigs submitted to the Animal Health Diagnostic Laboratory at Michigan State University by Michigan producers between 6-30-81 and 12-31-82. Only live pigs 2 weeks of age or younger with clinical evidence of diarrhea were used in this study. Pigs were euthanatized with T-61[n] administered intravenously. Necropsy was performed immediately following death to minimize autolytic postmortem changes. Ileal sections from these pigs were subjected to the indirect fluorescent antibody technique previously described, and the results were used to determine a prevalance of the K88, K99, and 987P pilus antigens of *E. coli* in Michigan neonatal pigs.

Quantitation of Lactose-Fermenting Bacteria

A 10 cm section of ileum was obtained from each pig included in this study. The ileal segment was ligated at each end with suture material. The tissue was placed in a 0.3% solution of peptone water[o] to give a total volume of 30 ml. This segment in peptone water was then blended at high

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[n]T-61 Euthanasia Solution, National Laboratories Corp, American Hoechst Corporation, Somerville, NJ.

[o]Bacto-Peptone, Difco, Detroit, MI.

speed for 1 minute in a Sorvall Omni-Mixer[p]. One milliliter of the resultant slurry was pipetted into 9 ml of peptone water, and eight 10-fold serial dilutions were made in triplicate. One milliliter of each dilution was cultured on MacConkey agar using a standard pour plate technique with incubation overnight at 37°C. An average colony count for each dilution was determined by counting viable colonies using a digital colony counter[q], adding the counts together for that dilution, and dividing by 3. Those dilutions in which the average count was between 30 and 300 were used in the calculation of the number of lactose-fermenting bacteria according to the following formula:

$$Con = 1/10^{-a} \times 30 \times b$$

where Con is the original concentration of the numbers of lactose-fermenting bacteria per 10 cm of ileum, 10^{-a} is the dilution from which the inoculum was taken, 30 is the total volume of peptone water into which the 10 cm segment was homogenized, and b represents the average number of colonies counted for that dilution. An unpaired "t" test was used to compare numbers of viable lactose-fermenting bacteria in pigs with and without piliated E. coli in their ilea (Steel and Torrie, 1960).

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[p]Ivan Sorvall, Inc., Newtown, CT.
[q]Lab-Line Digimatic Colony Counter, Lab-Line, Inc., Melrose Park, IL.

Association Index

The degree of association between piliated E. coli and intestinal epithelium was determined morphologically by a modified association index as described by Bertschinger et al. (1972). Giemsa-stained cross sections of ileum were examined microscopically with the 40x objective.

The association index was determined by 2 criteria. The first criterion was the number of bacteria seen at the base of villi, and this evaluation was given a value from 1 to 5. A value of 1 represented no bacteria at the villous base and a value of 5 represented maximal numbers of bacteria in this location. The second criterion was based on the tendency of these bacteria to be contiguous to epithelial cells, and was graded similarly from 1 to 5. The association index for an ileal segment was obtained by multiplying the values obtained from those 2 criteria. An association index value of 25 indicated maximal numbers of bacteria at the villous base with a strong tendency to be contiguous to the apical portions of epithelial cells. However, an index of 1 indicated that bacteria were lacking at the villous base and that bacteria were not contiguous to the intestinal epithelium. A sign test was used to determine statistical significance between pigs with and without piliated E. coli in their ilea (Steel and Torrie, 1960).

RESULTS

Control Animals

Clinical Signs

All gnotobiotic pigs appeared to be healthy prior to the administration of the control inocula. Within 24 hours after giving the inocula, pigs in isolators 2, 3, and 4 had evidence of diarrhea, and the pigs in isolator 3 had died. The pig in isolator 1 appeared normal. A summary of the pilus antigens of *E. coli* to which the pigs were exposed is seen in Table 3.

Gross Lesions

The pigs in isolators 1, 2, and 4 were euthanatized 24 hours after exposure. Necropsies were performed on all pigs. The pig in isolator 1 had a cyst on 1 lobe of the liver and mild edema of the spiral colon. Intestinal contents were normal. Pigs from isolators 2, 3, and 4 had watery contents in the small and large intestines. Also the wall of the small and large intestines appeared thinner than normal. Pigs from isolator 3 both had hyperemia of the serosal surface of the small intestine.

Microscopic Lesions

Sections of kidney, liver, and lung from the pig in isolator 1 were normal microscopically. However, sections of lymph node and spleen appeared decreased in cellularity. Sections of duodenum, jejunum, and ileum were normal in microscopic appearance, but the jejunum and ileum had vacuolated cytoplasm in their epithelial cells.

The pigs in isolators 2 and 4 had similar microscopic lesions. The kidneys, lungs, lymph nodes, and livers of these pigs were normal microscopically. The spleen appeared hypocellular in 1 of the pigs from isolator 2. No bacterial colonies were seen in Giemsa-stained sections of duodenum, but the jejunum had occasional rod-shaped organisms adherent to tips of villi. Vacuolation of the cytoplasm was also noted in the jejunum. The ileal epithelium was characterized by cytoplasmic vacuolation and greater numbers of adherent rod-shaped bacteria than seen in the jejunum.

The pigs in isolator 3 had a normal microscopic appearance in the kidneys, livers, lungs, and lymph nodes. The spleen appeared congested in 1 of these pigs and normal in the other. The gastrointestinal tract had autolysis in the duodenum, jejunum, and ileum, and cytoplasmic vacuolation was noted in the epithelial cells of those three levels. On Giemsa-stained sections, the duodenum and ileum had the most rod-shaped bacteria adherent to epithelial cell surfaces, and the jejunum had the fewest. Some of the villi

in sections of ileum from these pigs appeared shorter than normal.

Immunofluorescence

Ileal sections of control animals were used to determine the specificity of the indirect fluorescent antibody technique for demonstrating pilus antigens. Sections that had apple-green-colored fluorescence along the tips and lateral surfaces of the villi were considered positive for pilus antigens (Figure 1). Sections that lacked such fluorescence were considered negative for the 3 pilus antigens (Figure 2). In many sections, autofluorescent eosinophils were seen in the lamina propria (Figures 1 and 2). All 3 pilus antigens had a similar degree and distribution of fluorescence. The results of this test are summarized in Table 3.

Table 3 - Results of indirect fluorescent antibody test on control animals.

Isolator No.	Pigs Exposed To	Test Result			
		K88	K99	987P	Neg
1	Unexposed	-	-	-	+
2	987P	-	-	+	-
3	K88	+	-	-	-
4	K99	-	+	-	-

Bacteriology

Monitoring Germfree Isolators. No bacterial growth from isolators 1 and 3 could be demonstrated. However, isolators 2 and 4 yielded a gram positive coccus, which may have been a contaminant. This was probably caused by an undetected break in the isolator unit. This was not judged to alter the results of this experiment.

Quantitation of Control Inocula. One milliliter of each broth culture of *E. coli* given to gnotobiotic pigs contained approximately 10^9 *E. coli*. The control inoculum administered to the pigs in isolator 1 yielded no bacterial growth (Table 4).

Table 4 - Numbers of *E. coli* from control inocula.

Isolator	Strain of *E. coli* in control inoculum	Numbers of *E. coli*
1	Control	0
2	987	9[a]
3	I248	9
4	431	9

[a]Data expressed as mean $\log_{10}$.

Field Specimens

A total of 125 pigs from 55 Michigan swine herds were submitted to the Animal Health Diagnostic Laboratory between 6-30-81 and 12-31-82. Each herd had a history of diarrheal disease (Table 5).

Table 5 - Distribution of pigs submitted with a history of diarrhea.

No. of herds represented	No. of pigs examined	No. of pigs with diarrhea	No. of pigs without diarrhea
55	125	119	6

Clinical Signs

Of the 125 pigs in this study, 119 had clinical evidence of diarrhea. The feces were often yellowish in color and watery in consistency. Such pigs usually had sunken eyes and increased skin turgor as evidence of dehydration. Vomiting was rare.

Gross Lesions

The gross lesions of pigs submitted with a history of diarrhea varied considerably. A reddening of the skin in the perianal area was common because of constant irritation due to watery feces. The small and large intestines were often hyperemic on the serosal surface. Several pigs had edema of the mesentery of the spiral colon. Mesenteric

lymph nodes were occasionally swollen. However, none of these lesions could be considered pathognomonic for 1 etiologic agent.

Microscopic Lesions

Observation of microscopic lesions for this study was limited to sections of ilea from pigs submitted with a history of diarrhea. The nature of the ileal lesions usually varied depending upon the etiologic agents isolated from the various tissue specimens routinely examined. For example, pigs with coronavirus infection were often observed to have blunted, atrophic villi in certain sections of ileum. Those pigs in which a piliated *E. coli* was demonstrated had ilea in which rod-shaped bacteria could often be seen adhering to the tips and lateral surfaces of villi. This was best seen by using a Giemsa stain (Figure 3).

Prevalence of Pilus Antigens

The K99 pilus antigen was the most frequently encountered in this study (23 of 125), and the K88 (14 of 125) and 987P (13 of 125) pili were encountered less frequently (Table 6). In 2 of the 125 pigs examined, both K88 and K99 pilus antigens were present simultaneously. In 45 specimens, a piliated *E. coli* was judged to be the only enteropathogen responsible for diarrheal disease. In 7 other cases, a piliated *E. coli* concomittant with another

enteropathogen was responsible for enteric disease. In 37 specimens, pathogens of the gastrointestinal tract other than piliated E. coli were judged to be the cause of diarrhea. Of these 37, coronavirus was demonstrated in 18 pigs, coccidiosis was diagnosed in 3 pigs, and other causes of disease were felt to be present in 16 pigs. This category included septicemias due to Streptococcus sp., milk production problems in the sow, or nutritional problems in the herd in question. In the remaining 36 pigs examined, no enteropathogen could be definitively identified to be the cause of intestinal disease. (Table 7).

Table 6 - Numbers of pigs, diagnoses made, and E. coli pili identified in pigs submitted with a history of diarrhea.

Diagnoses made	Pili identified			
Colibacillosis only	K88	K99	987P	K88 & K99
45	11	21	12	1
Mixed infections with colibacillosis				
7	1[a],2[c]	2[b]	1[c]	1[a]
Total				
52	14	23	13	2

[a]Colibacillosis and rotavirus.
[b]Colibacillosis and coronavirus.
[c]Colibacillosis and salmonellosis.

Table 7 - Numbers of pigs with diagnoses other than piliated E. coli infection submitted with a history of diarrhea.

Coronavirus	Coccidiosis	Other[a]	Undetermined
18	3	16	36

[a]Includes septicemias, milk production problems, or nutritional imbalances.

The number of Michigan swine herds with piliated E. coli as a cause of enteric disease was 27 of 55 herds examined as determined by the indirect fluorescent antibody technique. The numbers of herds in which pili of E. coli were identified are summarized in Table 8. The total number of individual pigs testing positively for piliated E. coli was 52 of 125 pigs examined. On a herd basis, this represented a prevalence of 49%, and on an individual basis the prevalence was 42% (Table 9).

Table 8 - Numbers of herds in which pili of E. coli were identified.

K88	K99	987P	K88 & K99	K88 & 987P
7	11	5	2[a]	2[b]

[a]Both pilus antigens were found simultaneously in two pigs from different herds.

[b]Pilus antigens were not found simultaneously but were found in different submissions from these 2 herds.

Table 9 - Prevalence of piliated *E. coli* in swine herds and pigs in this study.

Herds with piliated *E. coli*	Pigs with piliated *E. coli*
27/55[a] (49%)	52/125[a] (42%)

[a]Number positive/number tested.

Quantitation of Lactose-Fermenting Bacteria

Pigs in which piliated *E. coli* were identified had a significantly higher number of lactose-fermenting bacteria present in the ileum ($p<0.05$) than did pigs in which piliated *E. coli* were not identified (Table 10). Biochemical tests on selected colonies from 10 pigs revealed *E. coli*; however, bioassays for enterotoxigenicity were not performed.

Table 10 - Numbers of lactose-fermenting bacteria in 10-cm loops of ilea in pigs with and without piliated *E. coli*.

	No. of pigs	Mean[a]	S.E.M.[b]	p[c] value
With piliated *E. coli*	52	7.4	0.3	
Without piliated *E. coli*	73	5.6	0.3	$p<0.05$

[a]Data expressed as mean $\log_{10}$/10 cm of ileal segment.
[b]Standard error of the mean.
[c]Probability of no difference between the means.

Association Indices

In those pigs in which a pilus antigen of *E. coli* was identified, the mean association index was 12.1. In those pigs in which no pilus antigens were identified, the mean association index was 1.9. Statisitical analysis by the sign test demonstrated a significant difference between these two groups (Table 11).

Table 11 - Association indices of bacterial adhesion in pigs with and without piliated *E. coli*.

	Mean	Range	Mode	P value[a]
With piliated *E. coli*	12.1	2-25	12	P<0.05
Without piliated *E. coli*	1.9	1-20	1	

[a]Probability of no difference between the means.

Figure 1. Photomicrograph of a fluorescent antibody-stained section of ileum from a control pig administered *E. coli* bearing K99. Positive, specific fluorescence for the K99 pilus antigen is located along the tips and lateral surfaces of villi. Eosinophils in the lamina propria have nonspecific autofluorescence (160X).

Figure 2. Photomicrograph of a fluorescent antibody-stained section of ileum from a control pig inoculated with trypticase soy broth only. Positive, specific fluorescence is lacking. Eosinophils in the lamina propria have nonspecific autofluorescence (160X).

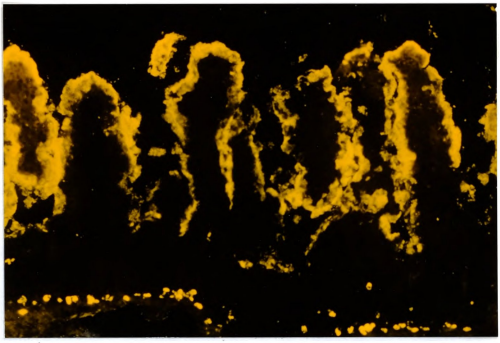

Figure 1

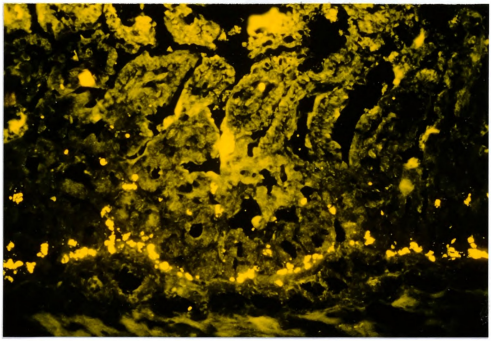

Figure 2

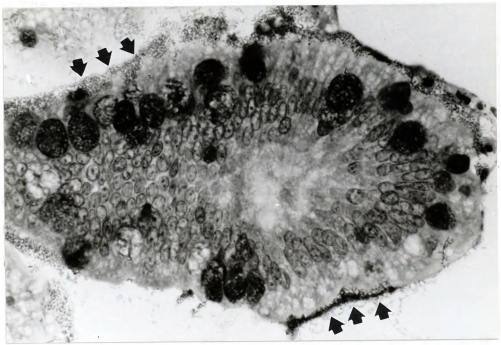

Figure 3. Photomicrograph of a section of ileum from a pig submitted with a history of diarrhea. Rod-shaped bacteria (arrows) are adherent to the tips and lateral surfaces of villi (Giemsa stain, 1000X).

DISCUSSION

The increased vacuolation of the cytoplasm in sections of the intestinal epithelium was believed to be caused by the lack of normal epithelial cell turnover, often seen in the gnotobiotic pig in which normal gut flora is absent (Moon, 1972). The hypocellularity of certain lymphoid organs may be due to lack of antigenic stimulation which may be seen in the young animal. These changes were felt to be consistent with the gnotobiotic environment in which these pigs were maintained.

The indirect fluorescent antibody technique applied to intestinal sections proved to be a rapid and effective method for the diagnosis of colibacillosis caused by piliated Escherichia coli in pigs. Other workers have demonstrated that this technique is preferable to the slide agglutination test (Moon, 1978b; Isaacson et al., 1978b) due to the fact that piliated E. coli enter a nonpiliated phase when cultured in some kinds of artificial media (Ørskov and Ørskov, 1966; Ørskov et al., 1975). The indirect fluorescent antibody method detects the organisms in their piliated phase and is, therefore, a more reliable diagnostic tool.

The results of this study indicated that piliated E. coli are responsible for diarrheal disease in Michigan neonatal pigs. The frequency with which the 3 pilus

antigens were found was approximately equal. However, the K99 pilus was encountered slightly more often than K88 or 987P. This is in contrast with results from another porcine study encompassing the north central United States in which the K88 pilus antigen was encountered most frequently (Francis, 1982). This study included pigs with diarrhea that were younger and older than 2 weeks of age, and this fact alone may have been responsible for the K88 pilus being most commonly found. It has been speculated that K88 is more common in the weanling pig, whereas 987P tends to be found in pigs less than 1 week of age (Francis, 1982). Perhaps the fact that the Michigan study excluded pigs beyond 2 weeks of age accounted for the lower than expected numbers of pigs with E. coli bearing the K88 pilus. It is of interest that 10 of the 13 pigs with 987P in our study were 1 week of age or less.

The fact that the K99 pilus antigen was most frequently encountered in this study may be due to the relatively small number of pigs available for this study. Perhaps if more pigs with colibacillosis were available for pilus antigen determination, a different prevalence pattern may have emerged. Another possible explanation may be that many of the pigs examined were from small farms in which other livestock, including cattle, were present. Because E. coli bearing K99 is known to be infective for cattle, perhaps the presence of cattle and pigs on the same premises is reponsible for our observation that K99 was the most commonly

encountered pilus antigen. Nevertheless, the results of this and other studies (Francis, 1982; Moon, 1978b; Ørskov et al., 1961) justify the use of trivalent vaccines to prevent the intestinal colonization in neonatal pigs of E. coli bearing K88, K99, or 987P.

In diarrheic pigs from the same farm there was a tendency for the same pilus antigen to be detected from all pigs. For example, a single submission of 5 pigs from the same owner yielded E. coli bearing K99 (See Appendix). Another submission of 3 pigs from a different farm all had E. coli with 987P. This same observation tended to be true for pigs submitted within the same litter.

Pigs with other enteropathogens (rotavirus, coronavirus, or Salmonella sp.) concomittant with piliated E. coli were not necessarily colonized with one particular pilus type. The K88, K99, or 987P pili were found simultaneously with other infectious agents known to cause enteric disease in the pig. The distributions of E. coli with each of the 3 pili were nearly the same in mixed infections as in those infections involving exclusively E. coli. This observation is in agreement with a study by Francis (1982).

Those specimens in which 2 pilus antigens were present in the same sections of ileum are of particular interest. Apparently, more than 1 strain of piliated E. coli may colonize the small intestine simultaneously. These specimens probably represent 2 different strains of

enterotoxigenic E. coli, each bearing a different pilus antigen rather than 1 strain of the organism with 2 different pili on each bacterium (Moon, 1982). However, it has been reported by Schneider and To (1982) that 2 enterotoxigenic strains of E. coli were both found to express 2 different pilus antigens at different times. This indicates that some field isolates of E. coli have the potential to produce more than 1 type of pilus antigen that promotes intestinal colonization, but it has not been rigorously established if such a strain produces 2 pilus antigens simultaneously.

In pigs in which piliated E. coli were identified, the numbers of bacteria able to ferment lactose were sometimes lower than expected. Some workers have quantitatively defined intestinal colonization in the pig by enterotoxigenic E. coli (ETEC) as 10^8 ETEC per 10 cm segment of ileum (Moon et al., 1979). The fact that total numbers of lactose-fermenters per 10 cm of ileum in pigs harboring E. coli with pilus antigens was sometimes less than 10^8 may have been associated with the administration of oral antibacterial agents to pigs prior to submission to the Animal Health Diagnostic Laboratory. This same factor also could cause a false negative fluorescent antibody result if numbers of piliated E. coli were substantially diminished in those areas of the ileum from which sections were taken for fluorescent antibody determinations.

Some pigs that tested negatively for pilus antigens gave a higher than expected association index value. Such cases may represent E. coli with pilus antigens that are previously uncharacterized. These antigens would not be detected by our fluorescent antibody technique. Evidence for new, uncharacterized pili of E. coli is increasing (Moon, 1978b; Morris et al., 1982). Adhesins other than pili may also account for these results, but bacterial adherence by mechanisms other than pili has not been well elucidated.

Moon (1978b) has discussed the fact that E. coli bearing K88 tend to colonize both the upper and lower small intestine, whereas E. coli with K99 or 987P colonize only the lower half of the small intestine. The observations in our control pigs concurred with this, as the pigs exposed to E. coli bearing K88 (strain I248) were observed to have rod-shaped bacteria adherent to the sides and tips of villi when Giemsa-stained ileal and duodenal sections were viewed microscopically. However, duodenal sections from those pigs administered E. coli bearing K99 or 987P pili lacked such adherent bacteria.

Some workers have associated more virulence with those strains of E. coli bearing the K88 pilus than with those bearing K99 or 987P (Moon, 1982). The fact that our only deaths among control animals were those administered K88-bearing E. coli supports this contention. These animals died less than 18 hours after administration of control inocula. Wilson (1982) recently demonstrated that some E.

coli with K88 pili produced both LT and ST, but strains with either K99 or 987P produced only ST. The observations that enterotoxigenic E. coli bearing the K88 pilus produce 2 types of enterotoxins and colonize a greater length of the small intestine may confer increased virulence to those strains and may explain the deaths in our control animals. It may also explain the lower prevalence of K88 in this study, as only pigs submitted alive were used for pilus antigen determination. Perhaps if dead pigs with histories of diarrhea had been included, K88 may have occurred more frequently. More studies correlating virulence with toxin type and with type of pili expressed need to be conducted.

SUMMARY

A study of 125 pigs 2 weeks of age or less with histories of diarrhea was performed to determine the prevalence of the 3 pilus antigens of Escherichia coli (K88, K99, and 987P). An indirect fluorescent antibody technique on frozen ileal sections was utilized. Similar sections of intestinal tissue from gnotobiotic pigs orally infected with pure cultures of E. coli of known pilus type were used for controls. Sections of ileum 10 cm in length were used to determine total numbers of lactose-fermenting bacteria in pigs with and without piliated E. coli as determined by our indirect fluorescent antibody technique.

The results of this study indicate that the indirect fluorescent antibody technique is a rapid and sensitive diagnostic tool for the detection of K88, K99, and 987P pilus antigens of E. coli. Pigs in which piliated E. coli were identified had a significantly higher number of lactose-fermenting bacteria ($P < 0.05$) in 10 cm sections of ileum than did pigs in which piliated E. coli were not detected. In addition, all 3 pilus antigens of E. coli were found in Michigan neonatal pigs with diarrheal disease. The K99 pilus antigen was encountered slightly more often than K88 or 987P. These findings justify the use of trivalent

pilus vaccines containing purified K88, K99, and 987P pilus antigens to decrease the incidence and severity of enteric disease caused by enterotoxigenic E. coli.

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VITA

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In 1981, the author came to Michigan State University where he began a research project with Dr. Glenn L. Waxler on colibacillosis in neonatal pigs. The author is currently pursuing doctoral studies at Michigan State University.

APPENDIX

Preparation of Antibodies

The monospecific anti-pilus antibodies were prepared using 8 New Zealand White rabbits. Preparations of purified pilus antigens K88, K99, and 987P were obtained from Dr. R. E. Isaacson, School of Public Health, University of Michigan, Ann Arbor. Rabbits were divided into 4 groups, with 1 group serving as noninjected controls (Table A1). Antigens were received as suspensions in varying concentrations. Each injected rabbit received a volume containing 250 μg of antigen subcutaneously with an equal volume of complete Freund's adjuvant. The antigen and adjuvant were emulsified prior to injection. Not more than 0.2 ml was given per injection site. Injections were repeated again 4 weeks later. All rabbits were bled 7-10 days after the second injection. Blood was centrifuged, and the serum was removed. Serum was filtered through a 0.45 μm filter and stored in 10 aliquots of 2 ml each in a freezer at -70°C.

Table A1 - Distribution of rabbits injected with purified pili.

Rabbit number	Pilus injected
1	None
2	None
3	K99
4	K99
5	K88
6	K88
7	987P
8	987P

Data from Fluorescent Antibody Determinations

The data obtained in this study from individual pigs and herds are summarized in Table A2.

Table A2 - Data from fluorescent antibody determinations

AHDL Case No.[a]	Herd No.	Pilus Identified[b]
234992	1	None
235142	2	K99
235143	2	K99
235144	2	K99
235242	3	K88
235243	3	K88
235489	4	K88
235490	4	K88
235632	2	None
236152	5	K99
236153	5	K99
236363	6	None
236364	6	None
236564	7	987P
236734	8	K88
237315	9	None
237316	9	None
237317	10	None
238390	11	K99
238392	11	K99
238394	11	K99
239469	12	None
239471	12	None
240128	13	None
240507	14	None
241406	15	None
241407	15	None
242427	2	None
242635	16	None
242610	17	None
243773	18	K88
244002	19	None
245354	20	987P
245355	20	987P

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[a]Each Animal Health Diagnostic Laboratory case number represents an individual pig.

[b]As determined by the indirect fluorescent antibody technique used in this study.

AHDL Case No.	Herd No.	Pilus Identified
260588	21	None
260589	21	None
260590	21	None
260988	22	None
260989	22	None
261203	23	K88
263533	7	K88
263534	7	K88
266191	24	None
266192	24	None
267032	25	None
267033	25	None
269555	26	None
269556	26	None
272951	27	None
272952	27	None
273169	28	None
275189	29	None
278087	30	None
278088	30	None
278089	30	None
278927	31	None
278928	31	None
278975	32	None
278981	19	None
278982	19	None
279616	30	None
279795	25	K99
279796	25	K99
279797	25	K99
279798	25	K99
279799	25	K99
282606	33	None
282607	33	None
282997	34	None
282998	34	None
286367	35	K99
286368	35	K99
292502	34	987P
292503	34	987P
292504	34	987P
292505	34	None
293536	36	K88
293537	36	K88
293583	36	None
295054	3	None
295721	37	987P
295909	38	None
297708	39	K99
297709	39	None
301362	40	None

AHDL Case No.	Herd No.	Pilus Identified
301363	40	None
301364	40	None
301365	40	None
301366	40	None
301367	40	K99
301368	40	None
301544	41	K88 & K99
301545	41	K88
302214	42	K88
302215	42	None
303812	43	None
304415	44	K99
304416	44	K99
308147	45	None
309594	43	None
309882	46	None
309883	46	None
309884	46	None
310925	47	None
310926	47	None
310927	47	None
311808	48	K88
312316	49	K99
315323	50	K99
315324	50	K99
320149	51	987P
320150	51	987P
320151	51	None
320779	52	K99
320780	52	None
320781	52	None
320782	52	None
320952	53	None
320953	53	None
321138	54	K88 & K99
327155	36	987P
327156	36	None
327677	55	987P
327678	55	987P
327679	55	987P