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RESPONSE OF THE BOVINE FETUS TO *IN UTERO* VACCINATION WITH PURIFIED K99 PILI OF *ESCHERICHIA COLI*

presented by

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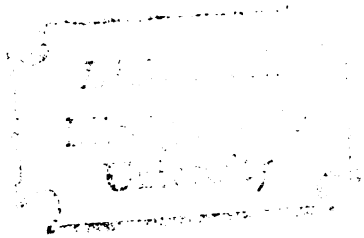

RESPONSE OF THE BOVINE FETUS TO *IN UTERO* VACCINATION WITH PURIFIED K99 PILI OF *ESCHERICHIA COLI*

By

Thomas P. Mullaney

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ABSTRACT

RESPONSE OF THE BOVINE FETUS TO *IN UTERO* VACCINATION WITH PURIFIED K99 PILI OF *ESCHERICHIA COLI*

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The response of the bovine fetus to intrauterine (oral) inoculation with purified K99 pili of *Escherichia coli* was determined by inoculating 12 cows at approximately 7 months of gestation with purified pili or sterile physiological saline. At birth calves were colostrum-deprived, placed in plastic-film isolators and fed autoclaved whole milk.

Control cows maintained normal pregnancies while 5 of 8 vaccinated cows aborted or had premature live calves. Control and vaccinated calves were orally challenged at 4 hours of age with 10^{11} K99 positive enterotoxigenic *E. coli* (ETEC) and all except 1 vaccinated calf died 24 to 48 hours following challenge. Immunofluorescent examination of ileum for K99 was positive in calves that died following challenge.

K99 pilus-specific antibody values measured by enzyme-linked immunosorbent assay (ELISA) were increased after vaccination with pili in sera from calves at birth and in intestinal secretions from calves at necropsy. Vaccination did not affect antibody values in sera from cows at parturition in comparison to antibody values from cows at vaccination. Antibody values were not increased in colostrum from vaccinated cows. Pilus-specific IgG_1 predominated in sera from

vaccinated calves, while IgA was the predominant antibody in intestinal secretions.

These results indicate the ability of the prenatal calf to respond to oral vaccination with purified K99 pili by pilus-specific antibody production in sera and intestinal secretions. Vaccination with pili, however, did not prevent fatal diarrhea caused by ETEC. Abortions and premature birth of calves following vaccination preclude widespread use of this technique.

DEDICATED TO MY MOTHER

MARGARET

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INTRODUCTION

Neonatal diseases of calves are a widespread problem of great economic importance. The economic impact of neonatal disease of calves to the cattle industry has been investigated. House (1978) estimated that calf losses in the United States between 1970 and 1976 were in excess of $95 million per year. *Escherichia coli*-induced diarrheal disease was associated with the majority of losses.

Diarrheal disease of neonatal calves has a complex etiology. Numerous agents, both infectious and non-infectious, have been implicated in the syndrome (Moon et al., 1976). Enterotoxigenic *E. coli* (ETEC), rotavirus, coronavirus and a coccidium of the genus *Cryptosporidium,* either alone or in combination, are regarded by researchers to be responsible for the greatest losses (Moon et al., 1978; Morin et al., 1978). Other agents, including adenovirus, parvovirus, infectious bovine rhinotracheitis virus, bovine virus diarrhea virus, astrovirus, calicivirus, "fringed particles", *Chlamydia sp.,* and *Campylobacter sp.,* are mentioned in the literature as possible causes of neonatal diarrhea of calves, but their role in the pathogenesis of the syndrome has not been elucidated (Curtis et al., 1966; Lambert et al., 1969; Storz et al., 1971; Mattson, 1973; Storz and Bates, 1973; Bulmer et al., 1975; Tzipori, 1981). Non-infectious predisposing factors such as overfeeding, overcrowding, chilling, dampness, poor hygiene and the use of milk replacers complicate the etiology even further.

Because of the complicated etiology of enteric disease in neonatal calves, it is not surprising that for many years little progress was made on enteric disease research. Diagnosis was a major problem because of the presence of multiple enteropathogens. In addition, although *E. coli* were associated with diarrheal disease of calves for almost one hundred years, the significance of *E. coli* isolates from the intestine was difficult to interpret because *E. coli* are part of the normal flora of the gastrointestinal tract. Accordingly, many researchers addressed themselves to the diagnostic problem of differentiating enteropathogenic *E. coli* (EPEC) from non-EPEC (Sojka, 1965; Smith and Halls, 1967a). Early research demonstrated the role of colostrum in prevention of death in neonatal calves (Smith and Little, 1922). Various treatments were evaluated (Radostits et al., 1975), but it is generally agreed that emphasis on sulfonamides and antibiotics is not the answer in preventing the problem. *Escherichia coli* vaccines were frequently used and results were both favorable (Myers et al., 1973; Newman et al., 1973) and unfavorable (Acres and Radostits, 1976). Rutter and Jones (1973) attributed unsatisfactory vaccination results to "incomplete knowledge of both the pathogenesis of intestinal infections and the protective immune responses of the alimentary tract, with the result that vaccine development has been largely empirical." Despite all this research, it seemed that significant progress had not occurred because Martin et al. (1975), after an extensive study of factors related to calf mortality in California, concluded "there has been no published evidence to indicate a decrease in the rate of calf mortality since the turn of the century."

However, about this time some exciting new developments were in progress. Bacterial pili were discovered in 1949 when the

ultrastructure of bacteria was examined using the electron microscope by independent researchers Anderson and Houwink (cited by Ottow, 1975). During the next 20 years reports of basic research on pili were frequently published (reviewed by Ottow, 1975). With the discovery of the role of certain pili as colonization factors in ETEC induced diarrheal disease in pigs (Jones and Rutter, 1972; Smith and Linggood, 1971) and in calves (Moon et al., 1979a; Smith and Linggood, 1972), the pathogenesis of the disease was more fully elucidated. Colonization of the small intestine is one essential step in the pathogenesis of ETEC induced diarrheal disease. This means that certain pili are virulence attributes of ETEC. The pilus which is associated with a very high percentage of ETEC strains in calves was designated K99 (Ørskov et al., 1975). Three different pili are currently known to be associated with ETEC strains in pigs and are designated K88ab or K88ac (Ørskov et al., 1964), K99 (Moon et al., 1977) and 987P (Isaacson et al., 1977a). Furthermore, basic research on pili had practical application when it was demonstrated that vaccination with a virulence determinant such as the K88 antigen of *E. coli* provided protection against ETEC-induced diarrheal disease in pigs (Rutter and Jones, 1973).

K99 pili were used as experimental vaccines in pregnant cows and results were promising (Acres et al., 1979; Nagy, 1980). There was a high correlation between protection of calves following oral challenge with K99 positive ETEC and the presence of K99 pilus-specific antibodies in colostrum (Acres et al., 1979). The success of this form of passive protection is dependent on good management, as calves must receive adequate colostrum soon after birth.

A different approach which has stimulated considerable interest in prevention of ETEC-induced diarrheal disease was to establish an active immune response in the prenatal calf by the use of *E. coli* bacterins as *in utero* vaccines (Gay, 1975; Olson and Waxler, 1976; Conner et al., 1973; Conner et al., 1977). These vaccines have usually protected calves orally challenged with homologous *E. coli* strains at birth (Gay, 1975; Conner et al., 1973). However, the occurrence of abortions and premature births has prevented widespread use of this technique. It has been suggested that abortions following *in utero* vaccination may be induced by endotoxin in the vaccine (Conner et al., 1973; Gay, 1975).

This research investigated prevention of ETEC-induced diarrheal disease by determining the response of calves to *in utero* vaccination with purified K99 pili. The hypothesis was that since purified K99 pili preparation contains no cell wall material and consequently very little endotoxin, it might be a suitable antigen for use as an *in utero* vaccine. The response to vaccination was determined by oral challenge of colostrum deprived calves with K99 positive ETEC. The antibody response to vaccination was measured by determining K99 pilus-specific and isotype-specific antibody titers in calf sera and intestinal secretions.

LITERATURE REVIEW

The literature relating to neonatal diarrhea in calves is voluminous. This review will therefore concentrate on the literature pertinent to this research. After a brief history of *Escherichia coli* infections in animals, I will review clinical signs, pathogenesis, enterotoxins, pathology, mechanisms of diarrhea, bacterial pili and vaccination with pili associated with ETEC-induced diarrheal disease in calves. The remainder of the review will focus on bovine immunoglobulins and on the development and uses of immunologic procedures used in this research.

Escherichia coli Infections in Animals

Escherich in Germany in 1885 examined the feces of newborn babies and found that bacteria which he called "Bacterium coli commune" appeared in the feces in association with breast feeding (cited by Sojka, 1965). The organism described by Escherich is now generally accepted as being *Escherichia coli*. *Escherichia coli* is a normal inhibitant of the intestine of man and animals and as such its distribution is almost ubiquitous. It is a gram-negative, non-sporing bacillus, 2-3 μm in length and 0.6 μm in width. It is classified as a member of the family Enterobacteriaceae. Larguelle in 1889 was the first to suggest that *E. coli* was a potential pathogen (Sojka, 1965).

A condition known as "calf scours" or "white scours" because the feces are frequently white in color has been recognized as a common disease of neonatal calves since the 19th century. Jensen in 1893 was the first to associate *E. coli* with white scours of calves, or colibacillosis, as the condition is frequently referred to (Sojka, 1965). The first report of experimental reproduction of colibacillosis in calves was by Jensen in 1913 (cited by Gay, 1965). Mild diarrhea occurred after calves were fed milk containing cultures of *E. coli* isolated from dead calves. Others failed in attempts to reproduce the disease (reviewed by Gay, 1965). Contrasting results probably occurred because the pathogenesis of the disease was unknown and the factors associated with virulence of strains of *E. coli* were also unknown at that time. There are numerous reports in the early literature of diarrheal disease and *E. coli* isolates from calves. However, because techniques were not available to differentiate pathogenic *E. coli* from non-pathogens, Gay (1965) correctly suggested that "it is extremely difficult to assess the significance of an *E. coli* isolate from a specimen and there is even greater difficulty in assessing reports in the literature dealing with such isolations." Early reports on the etiology of diarrhea in calves are also questionable because prior to 1969, when rotaviruses were discovered as a cause of neonatal diarrhea in calves (Mebus et al., 1969), researchers and diagnostic laboratories rarely had the techniques and equipment necessary to rule out non-bacterial causes of neonatal diarrhea.

Colibacillosis is a general term used to describe diseases caused by *E. coli* in neonatal animals and man. There is general agreement in the literature that colibacillosis is one of the most important and widespread diseases of neonatal calves, lambs and pigs (Sojka, 1965).

In pigs the term colibacillosis refers not only to *E. coli* infection in piglets a few days old but is also used to refer to *E. coli* infection in unweaned pigs about 3 weeks old and to a syndrome known as "edema disease" which occurs in weaned pigs between 8 and 12 weeks of age. The term colibacillosis also refers to *E. coli* infections in birds. Infections such as mastitis and metritis caused by *E. coli* occur in adult animals, but the term colibacillosis generally does not refer to these diseases.

Gay (1965) divided colibacillosis of young calves into 3 forms based on clinical signs, location of bacteria and possible pathogenesis. The first of these forms was called septicemic colibacillosis. Researchers have had no difficulty reproducing this form of colibacillosis in colostrum deprived calves using invasive strains of *E. coli* (Gay, 1965). Clinically these calves develop sudden anorexia, increased pulse and respiratory rates, prostration and rapid death. Although diarrhea is sometimes observed clinically, colisepticemia is not a primary enteric disease. Gross and microscopic lesions are quite variable. In peracute and uncomplicated septicemia of newborn calves there may be very few lesions, and diagnosis is based on clinical signs and extraintestinal localization of *E. coli*. Petechial and ecchymotic hemorrhages of spleen, epicardium and endocardium and hemorrhagic lymph nodes are sometimes seen. In less acute cases where clinical signs have persisted for a number of days, there may be combinations of fibrinopurulent polyserositis, arthritis, meningitis, ophthalmitis and pyelonephritis (Moon, 1974).

The factors which predispose calves to colisepticemia are well documented. Entry of *E. coli* into the blood occurs most commonly through the intestine or umbilicus in neonates (Moon, 1974) and occasionally

via the respiratory tract and tonsils. Staley et al. (1969) considered the pinocytotic apparatus of intestinal epithelium of neonates an important route for *E. coli* to enter into or through epithelium. Fey and Margadant in 1961 (cited by Gay, 1965) found, after examination of 22 calves that died of naturally occurring colisepticemia, that all were either agammaglobulinemic or markedly hypogammaglobulinemic. These findings were verified repeatedly by numerous researchers and it is now generally agreed that colisepticemia is a disease of immunoglobulin-deficient calves (McEwan et al., 1970; Selman et al., 1970; Logan, 1974). The virulence attributes of septicemia-producing strains of *E. coli* must also be considered. Moon (1974), in a review of the pathogenesis of colisepticemia, stated that "immunoglobulin-deficient calves are so greatly predisposed that any strain of *E. coli* has some potential to cause this disease." Smith (1962) found that the bactericidal activity of the serum was important in the pathogenesis of colisepticemia because strains of *E. coli* which grew in pre-colostral serum were capable of causing colisepticemia whereas those which did not grow in this serum did not cause colisepticemia when given to colostrum-deprived calves. Based on these observations, Moon (1974) concluded that "septicemic strains have no known special attributes that are related to toxigenicity or tissue invasion, but rather they are pathogenic because of their capacity to survive and multiply in blood and tissue." The *E. coli* strains involved in colisepticemia do not produce enterotoxin (Smith and Halls, 1967a). It is therefore presumed, but not conclusively demonstrated, that endotoxin shock is responsible for the depression, prostration and death associated with colisepticemia (Moon, 1974).

Because the virulence attributes of enteropathogenic strains of *E. coli* are plasmid mediated, Smith (1974) and Smith and Huggins (1976) investigated whether septicemic strains have plasmid mediated characters which are important in the pathogenesis of septicemic colibacillosis. Two plasmid mediated factors which contributed to pathogenicity were found. One was designated "Vir" and the other was the already known plasmid that determines colicine V (Col V) production. The Vir plasmid controlled production of a heat-labile and acid-sensitive toxin which was lethal when given intravenously to rabbits, mice and chickens. The Vir plasmid is not widely distributed among septicemic strains of *E. coli*. Smith (1978) found that only 6 of 247 strains of *E. coli* isolated from cases of septicemia were Vir positive. All of the Vir positive strains were antibiotic sensitive. Smith (1978) hypothesized that "the possibility of Vir plasmids emerging that also code for antibiotic resistance would be an unfortunate development because the widespread use of antibiotics might result in this comparatively rare but dangerous plasmid becoming more prevalent in nature."

The Col V plasmid was much more widespread among septicemic strains of *E. coli* and strains possessing this plasmid appeared to have a greater capacity to invade tissues (Smith, 1978). Smith also stated that the greater ability of Col V positive strains to resist defense mechanisms could be due to a proteinaceous coat around the organisms that made them more resistant to phagocytosis and complement. Obviously, much more research is required on these newly found virulence attributes of septicemic strains of *E. coli*. The toxin produced by the Vir plasmid apparently caused death in laboratory animals due to cardiac failure (Smith, 1978). This toxin appears to be different from endotoxin. Therefore, the conclusion by Moon (1974), which stated that endotoxin

shock was probably responsible for the depression, prostration and death associated with colisepticemia, should now be revised to include the following statement. Depression, prostration and death associated with colisepticemia are probably caused by the toxin produced by the Vir plasmid of *E. coli* or are due to endotoxin shock or to a combination of these effects.

Gay (1965) divided the second form of colibacillosis in calves into 2 entities: an enteric-toxemic form and an enteric form. In the enteric-toxemic form, there is sudden collapse and rapid death. Bacteremia is not a feature of this form; instead, the *E. coli* are localized in the small intestine, where massive proliferation occurs, and they produce a toxin which is absorbed and acts on tissues other than the intestine. In the enteric form, diarrhea is the predominant clinical sign followed by anorexia and dehydration. The course of the disease is variable. Death from dehydration and anorexia may occur after several days or slow recovery may result, but recovered calves remain unthrifty. Smith and Halls (1968b) regarded the intestinal form of colibacillosis in calves as a single entity. However, Moon (1974), in a discussion of the pathogenesis of enteric diseases caused by *E. coli*, divided intestinal infection into 3 forms: enterotoxic, enterotoxemic, and local-invasive colibacillosis. The enterotoxic and enterotoxemic forms occur in calves, and the local-invasive form is important in man and probably also in laboratory animals. Moon (1974) referred to enteric colibacillosis as enterotoxic because the mechanisms by which *E. coli* cause enteric disease were better understood at that time compared to the time when Gay (1965) made the original designation. It would also appear that enterotoxemic colibacillosis described by Moon (1974) is similar to enteric-toxemic colibacillosis described by

Gay (1965). However, Moon used edema disease of swine as the prototype for enterotoxemic colibacillosis. It can be argued that in calves a disease exactly similar to edema disease of swine does not occur. However, it would appear, based on pathogenesis, that some of the mechanisms are similar and that the strains of *E. coli* involved have similar attributes of virulence. These virulence factors include ability to colonize the small intestine and production of a toxin which is absorbed into the circulation. This toxin has not been well characterized, but it appears to be distinct from both endotoxin and enterotoxin (Moon, 1974). The toxin has also been referred to as *E. coli* neurotoxin, or "edema disease principle." It has been observed in swine that some strains of *E. coli* can produce both the edema disease principle and enterotoxin and as a result can cause both the enterotoxic and enterotoxemic forms of colibacillosis (Smith and Halls, 1968a). By extraploation it would appear that some strains of *E. coli* could cause both forms of enteric colibacillosis in calves because the pathogenesis of enterotoxic colibacillosis in calves and pigs is similar.

The pathogenesis of enterotoxic colibacillosis in calves is now well documented. Involved are colonization of the small intestine (Smith and Halls, 1967a; Moon, 1974) and production of enterotoxin (Smith and Halls, 1967b). These two attributes of virulence will be reviewed in more detail under the headings of "K99 Antigen of *E. coli*" and "*E. coli* Enterotoxins."

Classification of *Escherichia coli*

Researchers recognized over 80 years ago that certain strains of *E. coli* were pathogenic, but they had no reliable method of differentiating pathogens from non-pathogens. Early attempts at classification

based on agglutination were inconclusive because masking surface antigens rendered many strains inagglutinable (Sojka, 1965). Kauffmann (1947) published the first reliable method of classification of *E. coli* based on serological typing of the 3 major antigens of the organism. These antigens are 1) somatic O antigens, 2) capsular K antigens and 3) flagellar H antigens. This method of classification was more reliable than previous methods because the masking effect of K antigens on O antigen agglutination by homologous sera was removed by heat.

Gay (1965) stated that the O antigens are not single antigens but rather they are composed of several antigenic components and are therefore called the O group antigens. Within the family Enterobacteriaceae there is much cross reactivity between O groups because different O groups share certain antigenic components. The O antigens, also referred to as endotoxins, are composed of a polysaccharide-phospholipid complex with a protein fraction (Sojka, 1965). They are part of the bacterial cell wall and are liberated on autolysis of the cell and also by the use of solvents, heating and rapid freezing and thawing. Serogroups that have been associated with colisepticemia include O78, O15, O115, O86 and O55 (Gay, 1965), while O8, O9, O20 and O101 have been associated with enterotoxigenic strains of *E. coli* (Sojka, 1971). However, problems with identification of calf enterotoxigenic strains by means of serotyping were encountered when it was discovered that strains of *E. coli* with similar serogroups were found in health calves (Guinée, unpublished observation; cited by Guinée et al., 1976).

The K antigens are thermolabile polysaccharides and constitute the surface capsule of *E. coli*. They have been divided into 3 groups, called L, B and A, based on heat susceptibility and differences in other

physical properties (Gay, 1965; Sojka, 1965; Ørskov et al., 1977). The L-type antigens are completely destroyed by heat at 100 C for 1 hour and their antigenicity is also lost. This treatment then allows O antigens on these strains to agglutinate in homologous O antisera. The B antigens are destroyed by similar heat treatment but they retain their antigenicity. The A-type antigens require 121 C for 2.5 hours before they are destroyed sufficiently to unmask O antigens, which subsequently become agglutinable (Gay, 1965). Recently, however, it was reported that some strains possessing the K-A-type antigen can lose the heat resistance of the capsule by mutation (Ørskov et al., 1977). This means that strains of the same OK serotype can be found in an animal either with or without the special heat resistance of the K antigen. There is little information in the literature on the association between polysaccharide K antigens and colibacillosis in calves.

Ørskov et al. (1961), in Denmark, described a new type of antigenic variation in *E. coli* K antigens. During serological analysis of *E. coli* strains isolated from edema disease and enteritis in swine, they discovered strains with more than one form of K antigen. In one strain a new K antigen, designated K88, was present together with the already known K antigen 85. In another strain K88 was present along with K87. Later on, when the morphology, genetics and mechanism of action of K88 became known, it was recognized that this antigen was a virulence factor of enteropathogenic *E. coli* in pigs and facilitated adhesion to and colonization of small intestine. The discovery of K88 therefore represented an important discovery in enteric disease research because it was the first recognized virulence factor of *E. coli*, and when its mechanism of action was discovered the pathogenesis of enterotoxigenic colibacillosis became more obvious.

Ørskov et al. (1964), in further study of the K88 antigen, found that it was present among strains of *E. coli* isolated from piglets in England and Germany. They also found that K88 existed in at least 2 forms, one of which was designated K88 ab and the other K88 ac. Other reports demonstrated that the K88 antigen determinant was episome (plasmid) mediated (Ørskov and Ørskov, 1966).

Stirm et al. (1967a), working in Denmark, found that K88 was easily released by K88 positive bacteria into a suspending aqueous medium, especially when heated to 60 or 65 C. They isolated K88 from these extracts by ultracentrifugation and isoelectric precipitation. The purified K88 (ab form) was found to be a pure protein containing all of the common amino acids except cysteine and cystine. It was also demonstrated that purified K88 was immunogenic when injected into rabbits (Stirm et al., 1967a). K88 was therefore the first K antigen of *E. coli* shown to be a protein. In a further report, Stirm et al. (1967b) studied the morphology of K88 under the electron microscope and found that it consisted of a fur of fine filaments (pili) on the surface of the bacteria. These pili were 0.1 to 1.5 μm in length and 70 to 110 Å in width. Jones and Rutter (1972) examined the role of the K88 antigen in the pathogenesis of diarrhea caused by *E. coli* in piglets and clearly demonstrated that K88 facilitated attachment of K88 positive strains of *E. coli* in the small intestine. The K88 negative strains failed to attach to the small intestine and consequently were of low virulence in conventionally reared piglets. They also demonstrated inhibition of attachment *in vitro* by K88 antisera. This indicated that antibodies directed against K88 could prevent adhesion of K88 antigen to receptors in pig intestine. Jones and Rutter also stated that "if similar mechanisms occur in other enteric diseases this could lead to

the development of effective vaccines." Researchers have explored this possibility in recent years, and this topic will be reviewed under the heading "Pilus Vaccines."

K99 Antigen of *E. coli*

During studies on calf and lamb enteropathogenic *E. coli* strains, Sojka discovered that although the strains had different O antigens they possessed a common K antigen (cited by Smith and Linggood, 1972). In further work on this common K antigen, Smith and Linggood designated the antigen "Kco" and demonstrated that it was transmissible and plasmid-mediated. They also concluded that the Kco antigen was involved in the pathogenic process because when the Kco plasmid was removed from enteropathogenic lamb strains, the organism failed to produce diarrhea. However, diarrhea resulted when the same strain was given to lambs after reintroduction of the Kco plasmid. In addition, when Smith and Linggood (1972) found that Kco positive strains proliferated to high concentration in the small intestine of lambs and Kco negative strains did not, they concluded that "it is conceivable that the common K antigen functions in lamb-enteropathogenic strains in the same manner as the K88 antigen may function in some pig enteropathogenic strains by facilitating adhesion to intestinal epithelium."

Following serological investigation of the Kco antigen by Ørskov et al. (1975) on 11 strains isolated from calves and lambs, the antigen was designated K99. This designation was accepted by the World Health Organization Collaborative Center for Reference and Research on *Escherichia*. Subsequent studies demonstrated that the K99 antigen was present on a high percentage of ETEC strains and on a low percentage of non-ETEC strains isolated from calves (Guinée et al., 1976; Myers and

Guinée, 1976; Moon et al., 1976; Isaacson et al., 1978a). However, it was also found that the K99 antigen was difficult to detect by slide agglutination and unreliable when strains suspected of being K99 positive were grown at 37 C on media designed for the isolation of Enterobacteriaceae. Guinée et al. (1976) reported that detection of K99 was especially difficult with strains of *E. coli* having capsular K antigens of the A variety. They attributed difficulty in detection of K99 to the fact that many strains possessing this antigen grew as mucoid, heavily encapsulated colonies. Demonstration of K99 was facilitated when strains were grown on Minca medium (minimal casein medium), which is a buffered semisynthetic medium (Guinée et al., 1976). This medium was further improved by the addition of 1% Iso Vitale X (Guinée et al., 1977). Isaacson et al. (1978b) found that, although Minca-Iso Vitale X agar was a tremendous improvement over previously used media, it lacked reproducibility. They further optimized the detection of K99 by growing strains on Minca-Iso Vitale X agar. Strains that were negative when tested for K99 were passaged daily for 4 days in trypticase soy broth with vigorous shaking. They were then regrown on Minca-Iso Vitale X agar and retested by slide agglutination for K99.

K99 was purified and partially characterized by Isaacson (1977), working at the National Animal Disease Center and later at the University of Michigan. It consisted mainly of protein with 0.6% carbohydrate and 6.6% lipid. The purified antigen consisted of 2 subunits, a major component with a molecular weight of 22,500 and a minor component with a molecular weight of 29,500. Under electron microscopic examination, rod-like structures were observed. When the material was concentrated, the rods tended to self-aggregate and appeared filamentous. When diluted, the rods had an average diameter of 8.4 nm and an average

length of 130 nm. Based on its surface location and rod-like structure, Isaacson concluded K99 was a pilus or pilus-like structure.

Initially it was thought that K99 was associated only with enteropathogenic strains of *E. coli* in calves and lambs (Smith and Linggood, 1972; Ørskov et al., 1975). However, Moon et al. (1977) found that K99 was present in some ETEC strains isolated from pigs and also that K99-positive ETEC isolated from calves colonized and adhered to villous epithelium in the small intestine of pigs.

Demonstration of K99 either by immunofluorescence examination of small intestine (Isaacson et al., 1978a) or by detection of K99 in fecal extracts by an enzyme-linked immunosorbent assay (Ellens et al., 1978) are now both recognized as valuable techniques for diagnosis of ETEC-induced diarrheal disease of calves.

Escherichia coli Enterotoxins

De and Chatterje (1953) found that the ligated rabbit intestine test helped determine the enteropathogenicity and elucidate the etiology of neonatal diarrhea in humans. Smith and Halls (1967a), working in England, used this technique to study strains of *E. coli* isolated from livestock. They found that calf and lamb strains which caused dilation of ligated intestinal loops were also enteropathogenic. For example, all strains which dilated intestinal loops from calves and lambs caused severe diarrhea when given orally to colostrum fed calves and lambs less than 20 hours old. The dilation-negative strains caused no ill effect following challenge. In animals which developed diarrhea following challenge, the *E. coli* proliferated to high numbers in the anterior small intestine. They also noted that dilation-positive strains failed to produce diarrhea in calves 48 to 96 hours old, which indicated that

age resistance develops in calves to ETEC at about 2 days of age. In conclusion, they stated that "for a strain of *E. coli* to cause diarrhea or bowel edema it must be able to proliferate in the anterior small intestine; to produce diarrhea it must, in addition, produce enterotoxin, the substance responsible for dilation of ligated intestine." This was obviously a tremendous advancement in understanding the mechanisms involved in the pathogenesis of *E. coli* induced diarrheal disease. The biggest drawback with the ligated intestine test was that it was unreliable when strains isolated from calves and lambs were tested in laboratory animals. The test was most reliable when strains were tested in the same animal species from which they were isolated. Later Smith and Halls (1967b) demonstrated that enterotoxin was present in greater amounts in culture medium than in the bodies of the bacterial cells. They also reported that the enterotoxin was destroyed by heat at 121 C for 30 minutes and could be prepared in concentrated form by acetone precipitation of cell-free culture fluids. The ability of strains to produce enterotoxin was demonstrated by Smith and Halls (1968c) to be transmissible. They designated the genetic factor involved "ENT". Later, enterotoxin production was shown to be mediated by a population of transmissible plasmids (Skerman et al., 1972). Gyles (1970), in studies on enterotoxins from pig enteropathogens, found that a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST) were produced. There is general agreement that LT is antigenic (Gyles and Barnum, 1969; Smith and Gyles, 1970; Gyles, 1970) and that ST is non-antigenic (Smith and Gyles, 1970; Gyles, 1970). Subsequently, the structure and mechanism of action of LT were studied in detail. It is composed of a single polypeptide chain, and the molecular weight is variable but usually exceeds 100,000 (Dorner et al., 1976; Evans et al., 1976). In addition,

LT has a marked resemblance to and is antigenically related to cholera toxin (Gyles, 1974; Klipstein and Engert, 1977). Both cholera toxin and LT cause activation of the membrane-bound enzyme adenylate cyclase in the epithelial cells of the small intestine, which converts adenosine 5'-triphosphate (ATP) to cyclic adenosine 3',5'-monophosphate (cAMP). *Escherichia coli* LT therefore appears to have a hormone-like action which elevates intracellular cAMP resulting in increased intestinal secretion by crypt epithelial cells. Recent reports indicated, however, that LT does not cause elevation of cAMP in pig intestine (Newsome et al., 1978; Hamilton et al., 1978). This indicates that further research is required on the mode of action of LT in various species.

Attempts at purification of *E. coli* ST have not been completely successful. This may be due to the variety of assay procedures used and also because recent reports indicate that different types of ST exist (Newsome et al., 1978; Guerrant et al., 1975). The properties of purified or partially purified ST were described recently (Alderete and Robertson, 1978; Stavric et al., 1978; Kapitany et al., 1978). Newsome et al. (1978) separated ST into 2 components called ST_A and ST_B based on solubility in methanol and differences in properties. Reports by Field et al. (1978) and Hughes et al. (1978) indicated ST caused activation of guanylate cyclase resulting in increased concentrations of cyclic guanosine 3',5'-monophosphate (cGMP). Others consider the cAMP/cGMP ratio an important mediating factor in intestinal secretion by ST (Newsome et al., 1978).

Following the discovery of enterotoxins, researchers became interested in their potential as antigens to provide antitoxic immunity against ETEC-induced diarrheal diseases. The LT is antigenic and some studies indicated antibodies directed against LT were beneficial

(Kohler, 1978). The ST is non-antigenic and is produced by a high percentage of ETEC strains in calves (Smith and Halls, 1967a; Isaacson et al., 1978a). Therefore, the general consensus among researchers is that antibodies directed against LT would provide either incomplete or no protection against ETEC-induced diarrheal disease.

A wide variety of tests are now available for the detection of enterotoxins. Tests for LT include *in vivo* assays, tissue culture assays and *in vitro* assays. The *in vivo* assays include the gut loop tests, which are most sensitive and reliable when isolates are tested in the same species from which they were originally isolated. The infant rabbit test (Thorne and Gorbach, 1978) is also used to assay LT, especially from human isolates. Also, a rabbit skin test is used based on the fact that LT has an effect on vascular permeability (Evans et al., 1973).

Chinese hamster ovary (CHO) and mouse Y-I adrenal cell culture lines respond morphologically by rounding and physiologically by increased secretion of steroids to LT. These assays are now widely used and are very sensitive in detecting *E. coli* LT (Donta and Smith, 1974; Guerrant et al., 1974). The *in vitro* tests which are now available all have an immunologic basis. These include the lysis inhibition test (Evans and Evans, 1977), radioimmunoassay (Greenberg et al., 1977) and an enzyme-linked immunosorbent assay (Yolken et al., 1977).

Fewer assays of ST are available, and they all involve *in vivo* systems. The commonly used tests include the gut loop tests and the infant mouse test. In the latter test, supernatant fluid is injected through the abdominal wall and into the stomachs of infant mice. Four hours later the mice are euthanatized and the fluid accumulation response quantitated (Giannella, 1976). Other methods used to assay ST include

the rat perfusion test (Klipstein et al., 1976) and the infant rabbit test (Gorbach and Khurana, 1972).

Mechanisms of Diarrhea

A wide variety of processes, both infectious and non-infectious, affect the gastrointestinal tract. The response of an animal to many disease processes affecting the intestinal tract is the excretion of fluid feces. Frequently the clinical manifestations of viral, bacterial and protozoal disease of the intestine are indistinguishable. However, the underlying mechanisms which cause diarrhea to occur vary greatly. This topic was the subject of an excellent review by Moon (1978a). He listed hypermotility, increased permeability, hypersecretion and malabsorption as the major mechanisms in the pathogenesis of diarrhea.

Regarding hypermotility, Moon stated "I am not aware of any evidence to indicate that hypermotility is the primary or even a major contributing functional derangement leading to diarrhea in any disease. Therefore, it is useful to consider mechanisms other than hypermotility when attempting to understand and manage diseases." However, there is some evidence that hypermotility may contribute to diarrhea in cholera. Mathias et al. (1976) found that cholera enterotoxin stimulated increased peristalsis in the rabbit small intestine. Similarly, in ETEC-induced diarrheal disease evidence is beginning to accumulate that the enterotoxins cause hypermotility. Moon et al. (1979b) found that the transit of dye in the small intestine of infant mice was accelerated in response to *E. coli* ST. Burns et al. (1978) also found that *E. coli* enterotoxins stimulated intestinal contractions. Despite these observations, hypermotility appears to be only a secondary effect of the enterotoxins, and hypersecretion plays a much more important role in ETEC-induced diarrheal disease than hypermotility.

The role of decreased motility as a predisposing factor in the pathogenesis of diarrhea deserves mention. It is generally agreed that motility in the small intestine of newborn animals is sluggish because of the "germfree" state which exists at birth (Moon et al., 1979b; Abrams and Bishop, 1967). This sluggish motility would appear to be one factor which facilitates colonization of the small intestine of neonates by enteropathogenic bacteria. Chilling is another factor which predisposes young animals, especially piglets, to ETEC-induced diarrheal disease (Armstrong and Cline, 1977). Moon et al. (1979b) investigated the effect of temperature on intestinal motility in infant mice. They found that intestinal transit tended to increase as temperature increased from 25 to 37 C. In addition, 3 strains of *E. coli* attained greater numbers in the small intestine of mice maintained at 20 C compared to mice maintained at 25 C. Therefore, decreased motility, either associated with the "germfree" state in newborn animals or with chilling, appears to predispose to colonization of the small intestine by ETEC. When colonization has occurred and enterotoxins exert their effect, hypermotility may contribute to diarrhea. It might be more useful in the future to state that intestinal motility, either when increased or decreased, contributes to diarrhea, rather than using hypermotility alone to define one of the mechanisms of diarrhea.

Increased permeability and malabsorption are mechanisms of diarrhea not associated with ETEC. However, they deserve mention here because one of these processes could enhance the virulence of ETEC (Mebus et al., 1971a), and also because mixed infections (Morin et al., 1978; Acres et al., 1975) frequently cause neonatal diarrhea of calves.

Massive quantities of fluid are absorbed and secreted each day through small pores in the junctions between epithelial cells of the

small intestine of normal calves (Moon, 1978a). Usually equilibrium is maintained or there is slight net absorption. Inflammation of the intestine can result in increased pore size, causing increased leakage into the lumen of the intestine. At one time it was thought that ETEC and *Vibrio cholerae* caused increased permeability but it now appears that this does not occur (Moon, 1978a). Intestinal diseases such as lymphangiectasia in dogs and paratuberculosis cause large increases in pore size, resulting in exudation of plasma proteins. These diseases, as well as other protein-losing enteropathies and hemorrhagic enteritis caused by *Clostridium perfringens* Type C, are examples of intestinal disease where increased membrane permeability results in diarrhea (Moon, 1978a).

Malabsorption is the most important mechanism of diarrhea in enteric viral diseases of neonates. In transmissible gastroenteritis (TGE) of swine, coronavirus diarrhea of calves and rotavirus diarrhea of swine and calves, the viruses multiply in and destroy mature villous absorptive cells, resulting in decreased absorptive capability. In addition, because the absorptive cells also have digestive functions, digestion is also impaired. Unabsorbed and undigested food accumulates in the lumen of the intestine, draws fluid into the lumen of the intestine due to the increased osmotic activity, and thus contributes to diarrhea. It is important that these mechanisms associated with viral diarrhea are understood, because it has been suggested that viral damage to the intestine enhances the virulence of *E. coli* (Mebus et al., 1971a).

In ETEC-induced diarrheal disease, hypersecretion occurs due to the action of enterotoxins. The mode of action of enterotoxins in inducing hypersecretion has been discussed. Hypersecretion occurs from an intact mucosal surface. This means that oral electrolyte therapy is much more

in ETEC-induced diarrheal disease than in virus-induced diarrheal disease where absorption is impaired. The secreted fluids are alkaline, isotonic when compared with serum, low in protein, calcium, and magnesium and high in sodium and bicarbonate (Moon, 1974; Moon, 1978a).

Terminology

It is apparent that various terms are used in the literature to describe different types of enteric disease caused by *E. coli*. As the mechanism of action of the virulence attributes of *E. coli* were recognized, the names of the diseases were changed to specify the mechanism involved. This subject was reviewed and clarified by Moon et al. (1979a). Neter (1965) used the term enteropathogenic *E. coli* (EEC or EPEC) to describe strains of *E. coli* that had the potential to cause diarrheal disease and not extraintestinal disease. Enteropathogenic *E. coli* possess pili which facilitate colonization of the intestine. Enterotoxigenic *E. coli* (ETEC) produce enterotoxin(s). Enterotoxigenic *E. coli* that also have the virulence attributes to colonize small intestine are EEC (Moon et al., 1979a). Enterotoxigenic *E. coli* that are also EEC cause a disease in neonatal animals and man called enterotoxic colibacillosis (Moon, 1974), previously referred to as enteric colibacillosis, *E. coli* diarrhea, cholera-like *E. coli* infection, and "traveler's diarrhea" in man. Enteropathogenic *E. coli* which may or may not be ETEC also cause a syndrome known as enterotoxemic colibacillosis (Moon, 1974). The word "toxemic" refers to a toxin (not enterotoxin or endotoxin) which is absorbed from the intestine and acts systemically. Enteropathogenic *E. coli* in man and some laboratory animals which do not produce enterotoxin but rather penetrate intestinal epithelial cells cause a disease referred to as local-invasive colibacillosis (Moon, 1974; DuPont et al., 1971).

Pathology of Enterotoxic Colibacillosis

Although the literature relating to enterotoxic colibacillosis in calves is voluminous, this disease was difficult to reproduce until the virulence attributes of the involved strains of *E. coli* were discovered and the pathogenesis of the disease elucidated. Gay et al. (1964) and Smith (1962) attempted to reproduce enterotoxic colibacillosis in colostrum-deprived calves, but the calves died of colisepticemia. Frequently, colostrum fed calves did not develop diarrhea when challenge inoculated (Smith, 1962; Gay, 1965). Barnum et al. (1967) reported that the intestines of calves with diarrhea and suspected colibacillosis were histologically normal. Moon et al. (1970) also found that pigs with enterotoxic colibacillosis frequently did not have histologic lesions in the intestine. In contrast, Osborne (1967) found that calves given *E. coli* serotypes orally developed hemorrhagic enteritis. Consequently, the pathology of enterotoxic colibacillosis in calves was not well documented until Pearson et al. (1978a), working in Northern Ireland, reproduced the disease without the complication of colisepticemia by intravenous administration of immunoglobulin M to colostrum-deprived calves. Infected calves developed severe diarrhea and passed feces flecked with blood-stained mucus within 24 hours. Gross lesions consisted of several petechial hemorrhages in the abomasum close to the pylorus. In the distal ileum, contents were fluid and there were occasional petechial hemorrhages in the mucosa. The contents of the colon were fluid and contained gas bubbles in contrast to control calves, where the colonic contents were yellow and soft. Histologic lesions were confined to the distal half or one-third of the small intestine. Villi in the terminal ileum of infected calves were about 60% shorter than villi in control calves. Many of the stunted villi were adherent

to each other at the tip and along their lateral borders. The villous epithelium was usually cuboidal, but columnar cells were frequently also present. Small areas of lamina propria were exposed to the lumen of the intestine due to occasional small foci of coagulative necrosis of epithelial cells. Neutrophils, located either in the lamina propria or on the villous surface, were always associated with such lesions. Occasionally, small numbers of neutrophils and macrophages were observed on the surface of epithelial cells in the distal small intestine. The submucosa of the distal ileum was thicker than normal due to edema and infiltration of neutrophils, lymphocytes and plasma cells. However, Morin et al. (1978) found only minimal changes in the intestines of calves with spontaneous enterotoxic colibacillosis. The villi of the jejunum and ileum in these calves were either normal or only slightly shorter than normal in length. Villous epithelial cells were subcolumnar in shape in some areas, and denudation was uncommon. Bacteria were adherent to villous epithelial cells in the same location, as described by Pearson et al. (1978a). Therefore, although descriptions of the lesions associated with enterotoxic colibacillosis have improved in recent years, discrepancies still exist between experimentally induced lesions and the lesions described in spontaneous field cases.

Fluid and Electrolyte Abnormalities in Enterotoxic Colibacillosis

Calves with enterotoxic colibacillosis lose excessive quantities of alkaline, electrolyte-rich fluid from the small intestine. The onset of profuse watery diarrhea is followed by dehydration, hemoconcentration, metabolic acidosis, electrolyte abnormalities and shock (Tennant et al., 1972; Phillips and Knox, 1969). Although the mechanisms of diarrhea are totally different in calves with virus diarrhea and enterotoxic

colibacillosis, Lewis and Phillips (1972) and Phillips and Lewis (1973) reported that similar fluid and electrolyte losses occur in both situations. Calves with enteric infections usually have increased PCV, RBC and hemoglobin (Schultz, 1971). However, in some severely dehydrated calves PCV was in the normal range (Tennant et al., 1972; Tennant et al., 1975). This was explained by the fact that PCV values vary widely in neonatal calves. In dehydrated calves where PCV was within normal limits, it was probable that it was markedly increased above the value that existed prior to the onset of diarrhea. Plasma protein concentration is usually increased in diarrheic calves due to decreased plasma volume. However, total plasma protein concentration usually does not correlate with PCV values due to the wide normal variation in plasma protein concentration. This variation is attributed to wide variation in γ-globulin concentration (Tennant et al., 1978) and also due to the fact that calves with acute diarrhea lose protein via the intestinal tract (Marsh et al., 1969).

Metabolic acidosis is an almost constant finding in calves with enterotoxic colibacillosis. This occurs primarily from a loss of bicarbonate ions (Tennant et al., 1972; Tennant et al., 1978). In addition, as dehydration develops, increased anaerobic glycolysis occurs associated with peripheral vasoconstriction, resulting in increased production of lactic acid. The lactic acid contributes to acidosis because the liver cannot use it in gluconeogenesis due to venous congestion (Phillips and Lewis, 1973). Also, decreased renal perfusion results in decreased hydrogen ion excretion by the kidneys. As hypovolemic shock develops, respiration is depressed, which means that respiratory compensation is inhibited (Phillips and Knox, 1969).

Hyperkalemia is a characteristic finding in severely dehydrated calves (Tennant et al., 1972; Tennant et al., 1978). This is due to decreased renal tubular excretion of K^+ and movement of K^+ from intracellular to extracellular fluid due to severe metabolic acidosis. Also, breakdown of tissue during tissue catabolism could contribute to hyperkalemia (Blaxter and Wood, 1958). Decreased intracellular K^+ combined with increased extracellular K^+ interferes with myocardial conductivity, resulting in bradycardia and heart block (Fisher, 1965; Fisher and McEwan, 1967). Thus, hyperkalemia is generally believed to be the cause of death in calves with severe diarrheal disease.

Immunity to Colibacillosis

Smith and Little (1922) first demonstrated that ingestion of colostrum protected calves against white scours and septicemia. Aschaffenburg et al. (1949) found that the protective action of colostrum was associated with the whey fraction. Later, Logan et al. (1974a) reported that pooled whey fed to calves at birth protected the majority from death due to colibacillosis. Fey and Margadant reported in 1961 (cited by Gay, 1965) that 97% of calves which died of colisepticemia (in natural outbreaks) were agammaglobulinemic. Logan and Penhale (1971a,b,c), Penhale et al. (1971) and Logan et al. (1974b) reported on a series of detailed experiments designed to investigate the immunity of the calf to colibacillosis. They found that serum IgM was the principal class of immunoglobulin which protected calves against colisepticemia. They also found that colostrum had a protective effect within the lumen of the intestine. In the intestine, colostral IgM, IgG and IgA each had a role in protection. However, each class of immunoglobulin alone provided less protection than colostrum. Allen

and Porter (1975) administered bacterial antigens into the lumen of the small intestine of preruminant calves and found that antibacterial activity was predominantly associated with IgA and that IgM also played a significant role.

Colostrum must be ingested very early in life to provide protection against colisepticemia. During the first 24 hours of life, the newborn calf absorbs large quantities of undegraded colostral immunoglobulins. This specialized absorptive capability appears to be optimum at birth and is reduced rapidly during the first 24 hours of life. The time of "closure" of this absorptive mechanism has not been determined, but for practical purposes calves should receive adequate colostrum within 6 hours of birth. The colostral immunoglobulins are absorbed into the columnar cells of the small intestine by pinocytosis and transferred across them in vacuoles (reviewed by Logan, 1974). They reach the blood via the lymphatic system. Brandon and Lascelles (1971) concluded that colostral immunoglobulin classes were absorbed non-selectively. In contrast, Penhale et al. (1973) found that 90% of the IgG was absorbed but only 59% of the IgM and 48% of the IgA were absorbed, indicating selective absorption.

The general consensus among researchers now is that colostrum given to calves in adequate amount soon after birth provides protection from colisepticemia (Johnston et al., 1977; Logan, 1974) and, although beneficial, it does not completely prevent enterotoxic colibacillosis (Johnston et al., 1977; Acres et al., 1979; Myers, 1978). Researchers have therefore tried to provide passive protection to calves by vaccination of their dams with various antigens of *E. coli* (Acres and Radostits, 1976; Myers, 1976; Myers et al., 1973; Newman et al., 1973). The results of this kind of vaccination were unpredictable until known

virulence determinants of enterotoxigenic *E. coli* were incorporated into vaccines. Myers (1978), in Montana, reported on the efficacy of 6 different *E. coli* vaccines. He found that only a whole cell bacterin which possessed the K99 antigen stimulated a level of protection significantly greater than controls.

The age of the calf is another extremely important factor in immunity to colibacillosis. Smith and Halls (1967a) found that calves greater than 2 days of age were resistant to enterotoxic colibacillosis, whereas younger calves were susceptible. This resistance does not appear to be antibody mediated, because Smith and Halls (1967a) found that colostrum-deprived calves greater than 48 hours of age were also resistant when challenge inoculated with virulent strains of *E. coli*. Factors which might contribute to susceptibility of neonatal calves include sluggish motility of the intestines at birth associated with the germfree state. This lack of motility could contribute to colonization (Moon, 1974). In pigs, the gastric pH at birth is relatively high and *E. coli* proliferate in the stomach and arrive in the intestine in large numbers (Smith and Jones, 1963). Since neonatal calves have essentially monogastric function, it is conceivable that high gastric pH could also contribute to susceptibility of calves at birth. Age resistance in calves was thought to be associated with resistance of the small intestine to adhesion. However, Runnels et al. (1980) demonstrated, using an *in vitro* system, that intestinal cells from 4-day-old calves were susceptible to adhesion. Therefore, the factors which contribute to age resistance in calves are not completely understood.

Age resistance to ETEC in pigs differs from that of calves. For example, K99 positive ETEC cause enterotoxic colibacillosis in neonatal pigs (Moon et al., 1977) but not in pigs after weaning (Runnels et al.,

1980). In contrast, K88-positive ETEC cause diarrheal disease in pigs both before and after weaning (Sojka, 1965). Genetic resistance of some swine to adhesion by K88-positive ETEC has also been observed (Sellwood et al., 1975; Sellwood, 1980).

The role of other infections on susceptibility to enterotoxic colibacillosis is a subject that requires much more research in the future. Mebus et al. (1971a) indicated that rotavirus infection in calves enhanced the virulence of ETEC. However, Pearson et al. (1978b) found that the effects of concurrent experimental infection of calves with ETEC and rotavirus were additive rather than synergistic.

Bacterial Pili

When the electron microscope was used to study bacterial morphology, filamentous appendages were discovered radiating from the surface of many bacteria (reviewed by Ottow, 1975). These filamentous appendages were later termed fimbriae (Duguid et al., 1955) or pili (Brinton, 1965). For the purposes of this review, these ultramicroscopic cell appendages are referred to as pili. Pili can be defined as any morphologically distinct filamentous appendages of bacterial cells other than flagella (Ottow, 1975). The term pilus is derived from the Latin word meaning hair or hair-like structure. They occur very widely on gram-negative bacteria and are also present on some gram-positive bacteria such as *Corynebacterium renale*. Pili differ from flagella because they are ultramicroscopic, less rigid, thinner, straighter and more numerous. Pili vary in length between 0.2 and 20 μm, and their width usually ranges between 30 and 140 Å. Pili may be divided into somatic pili and sex pili (Nagy et al., 1978). The somatic pili of *E. coli* are the subject of discussion here because of their role as adhesive organelles. They have been divided into either 5 (Brinton, 1965) or 6 (Duguid et al.,

1966) groups based primarily on morphology. These pili have a peritrichous or polar arrangement around the bacterial cell and may number up to 1000 per organism. In contrast, the sex pili are scarce and usually there are between 1 to 10 present on a bacterium.

Interest in the pili of *E. coli* grew enormously when it was discovered that the K88 antigen and the K99 antigen had pilus morphology (Stirm et al., 1967b; Isaacson, 1977). The role of these pili as adhesive factors which contribute to the virulence of enteropathogenic strains of *E. coli* is now widely recognized (Moon, 1978b; Moon et al., 1979a; Smith and Linggood, 1971; Jones and Rutter, 1972).

Pilus Vaccines

Smith and Linggood (1971) and Jones and Rutter (1972) demonstrated the role of the K88 antigen in the pathogenesis of enterotoxic colibacillosis in swine. This new information provided Rutter and Jones (1973) with the impetus to investigate the possibility that "K88 antibodies transmitted via the colostrum to the piglet's intestinal tract should reduce attachment of K88-positive enteropathogenic bacteria to the mucosa and render the organism less virulent." They immunized pregnant gilts with partially purified K88 antigens and found that mortality was significantly reduced in piglets when the newborn piglets were experimentally challenged with K88-positive, virulent *E. coli*. Nagy et al. (1976) provided further evidence that colostrum from K88 vaccinated sows was protective because it prevented adhesion of K88-positive *E. coli* to the small intestine of nursing piglets.

Later, Isaacson et al. (1977b) and Morgan et al. (1978) reported that vaccination of pregnant gilts with either purified K99 pili or 987P pili resulted in protection of colostrum-fed piglets against diarrheal disease caused by ETEC strains that possessed the same pili.

Piglets exposed to ETEC strains that possessed different pili were not protected. They concluded that protection of piglets was probably due to the presence of anti-pilus antibodies in ingested colostrum. These antibodies in the small intestine could have prevented or reversed bacterial adhesion or could have agglutinated or opsonized the ETEC (Isaacson et al., 1977b). Nagy et al. (1978) confirmed the previous reports that vaccination of pregnant gilts with purified pili from strain 987 protected suckling offspring against diarrhea caused by 987P-positive ETEC. Later, Isaacson et al. (1980) measured K99 and 987P pilus-specific antibody levels in colostrum of sows vaccinated with purified K99 or 987P pili and found that antibody levels correlated with protection against ETEC possessing the same pilus as the vaccine.

Acres et al. (1979) vaccinated pregnant cows with purified K99 pili and found that nursing calves were protected against fatal diarrhea caused by K99-positive ETEC strains. They also found significant correlation between protection and K99 antibody titers in colostrum. Nagy (1980) used a crude K99 extract as a vaccine in pregnant cows and found that it protected colostrum-fed calves against fatal diarrhea caused by K99-positive ETEC strains. Colostral anti-K99 titers were increased in vaccinated cows and appeared to exert the protective effect. Similarly, when lambs received colostrum from K99-vaccinated ewes they were protected from experimentally induced enterotoxic colibacillosis (Sojka et al., 1978). All of these reports indicated that pilus vaccines will probably be a safe and effective means of preventing fatal diarrhea caused by ETEC strains in calves, pigs and lambs in the future.

One of the problems associated with development of purified K99 pilus vaccines for commercial use is the fact that low yields of pili are obtained (Isaacson, 1977; Isaacson, 1980b). This means that K99

pilus vaccines could be expensive due to the large volumes of broth cultures required to make a small amount of vaccine. Therefore, researchers have begun to investigate factors affecting the production of K99 pili. Recently, increased aeration was found to increase the amount of K99 produced by K99-positive strains (Isaacson, 1980a). Glucose, pyruvate, arabinose and lactose all inhibited K99, whereas glycerol stimulated K99 expression. De Graff et al. (1980) recently reported that 10 times more K99 was produced by *E. coli* strains possessing the 0101 serogroup than 08, 09 or 020 serogroups. Knowledge of these factors will help optimize K99 production for vaccine purposes and will also optimize detection of K99 *in vitro* and thus increase the reliability of diagnostic tests.

The efficacy of pilus vaccines in prevention of other diseases has also been reported. Pugh et al. (1977) used a *Moraxella bovis* pilus vaccine in bovines and found that it protected against experimentally induced infectious bovine keratoconjunctivitis. Pilus vaccines prepared from strains of *Neisseria gonorrhea* have been evaluated in human volunteers. Results appear promising, but the vaccine's effectiveness has not yet been completely established (reviewed by Marx, 1980).

Future Expectations

It is generally agreed by researchers that the cholera toxin, *E. coli* LT and ST and the pilus antigens bind to specific receptors in the small intestine. Already it has been demonstrated that monosialosyl glycolipid G_{Mi} ganglioside appears to be the receptor for cholera toxin (reviewed by Keusch, 1979; Richards and Douglas, 1978). Evidence is also available that *E. coli* LT binds to a closely related receptor

(Richards and Douglas, 1978). The nature of the K88, K99 and 987P receptors is unknown, but extensive research is in progress to determine the structure of these receptors. Keusch (1979) hypothesized that when the nature of these receptors is known "receptor megatherapy" might have therapeutic implications. For example, if an artificial receptor were given orally it could act as a competitive inhibitor of either toxin or pilus antigens. A second possibility suggested by Keusch was that of "receptor blockade, in which a specific binding unit with no capacity to activate the effector mechanism is employed to occupy the available surface receptors." Another theory proposed by Keusch was that of receptor modification. He stated that "it may be possible to alter an intestinal receptor *in-vivo* by supplying an appropriate enzyme to modify or remove the key terminal or internal sugars. Alternatively, a substrate could be locally supplied to accomplish the same goal through covalent linkage to the surface oligosaccharide, using endogenous glycosyltransferases already present and available in the outer surface of the cell membrane."

This futuristic approach may or may not have practical application in the control of diarrheal diseases. However, such ideas are an indication of the extremely complex mechanisms which researchers must focus on in the future.

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) was first developed and described by Engvall and Perlmann (1971) and Engvall et al. (1971) in Sweden. The ELISA technique is a simple, rapid, safe and highly specific method for assay of either antigen or antibody. For antibody assay (Engvall and Perlmann, 1972) antigen is coated on a solid phase

and then allowed to react with antiserum. A variety of surfaces, including glass and polystyrene, may be used as a solid phase. After incubation and washing, enzyme-conjugated anti-immunoglobulin is added to the antigen-antibody complex and the reaction is allowed to proceed for about 2 hours. After washing, an enzyme substrate which gives a good color reaction is added and the reaction stopped 30 minutes to 2 hours later. The uptake of enzyme to the solid phase gives a measure of the amount of specific antibody in the serum. The ELISA techniques have recently been used to detect antibodies to a wide variety of infectious agents and appear useful in clinical diagnosis (Byrd et al., 1976; Saunders et al., 1977; Kraaijeveld et al., 1980; Potgieter et al., 1980; Barrett et al., 1980). Carlsson et al. (1976) reported that ELISA techniques were 10 to 100 times more sensitive than standard tube agglutination for detecting *Brucella abortus* antibodies. Other reports indicate that ELISA techniques are also highly specific (Ruppanner et al., 1980; Wisdom, 1976), but obviously high specificity is dependent on the purity of the antigen used in the system. Because ELISA techniques are as sensitive as radioimmunoassay (Voller et al., 1978), are safer, and do not require expensive counting instruments, many laboratories are now using this system for routine diagnostic tests.

Bovine Immunoglobulins

Bovine immunoglobulins appear to have similar physical, chemical and antigenic properties to human immunoglobulins. The classes of bovine immunoglobulins include immunoglobulins G (IgG), M (IgM), A (IgA) and E (IgE). There are 2 subclasses of IgG, designated IgG_1 and IgG_2.

Immunoglobulin G is the predominant immunoglobulin in bovine serum and lacteal secretions (Butler, 1969; Butler, 1973). In normal adult

serum, IgG_1 and IgG_2 are present in almost equal amounts (Duncan et al., 1972). However, IgG_1 is selectively transported to the udder from the circulation and is consequently the predominant immunoglobulin in bovine colostrum and milk (Butler, 1969). Immunoglobulin G molecules have a sedimentation coefficient of 7S and are arranged into a monomeric unit consisting of 2 heavy (H) and 2 light (L) chains.

Serum IgM levels are generally higher in the bovine species than in man (Butler, 1969). Partial concentration of IgM occurs in colostrum, but it is present only in trace amounts in most other secretions (Butler, 1973). Immunoglobulin M is produced early in a primary immune response and has an important role in complement fixation and as an agglutinating antibody (Butler, 1969). It consists of 5 subunits linked together by a polypeptide J chain. Each subunit has the same structural arrangement as an IgG molecule.

Immunoglobulin A levels in bovine serum are generally significantly lower than human serum levels (Butler, 1973). Secretory IgA is the predominant immunoglobulin in all exocrine secretions of the bovine except lacteal secretions (Duncan et al., 1972; Mach and Pahud, 1971). Immunoglobulin A exists in a series of forms held together by a polypeptide J chain. In bovine serum, IgA usually occurs as a dimer, whereas in human serum IgA exists as a monomeric unit (Mach and Pahud, 1971; Duncan et al., 1972). Secretory IgA has a dimer form and an additional non-immunoglobulin fragment, known as secretory component, which apparently protects the immunoglobulin against enzyme degradation (Porter, 1973a).

The bovine fetus is capable of immunoglobulin production (Schultz et al., 1970; Schultz et al., 1971; Schultz, 1973). Cells containing IgM and IgG have been observed in the bovine fetus not overtly stimulated

with antigens (Schultz et al., 1973), but IgA has not yet been identified in the bovine fetus (Schultz, 1973).

In utero Vaccination

Immunization of the fetus with a variety of antigens has been reported in the past decade (Gay, 1971, 1975; Richardson and Conner, 1972; Conner et al., 1973; Cegnar et al., 1975; Conner and Carter, 1975; Olson and Waxler, 1976, 1977; Newman, 1978). Initially, surgical techniques were used, and later non-surgical techniques were developed (Conner and Carter, 1975; Gay, 1975). This vaccination technique protected newborn calves from experimentally induced enterotoxigenic colibacillosis, rotavirus diarrhea and coronavirus diarrhea (Conner et al., 1973; Conner and Carter, 1975; Olson and Waxler, 1976, 1977; Newman et al., 1978). Abortions and premature births were a frequent, unfortunate occurrence following vaccination (Conner et al., 1973; Gay, 1975; Newman et al., 1978), while in other reports this problem was not encountered (Olson and Waxler, 1976, 1977). The cause of the abortions or premature births has not been determined.

Summary

Research in recent years has demonstrated that pathogenic strains of *E. coli* possess plasmid-mediated virulence attributes. The important virulence attributes on ETEC strains in calves are the K99 pilus antigen and the enterotoxins (ST and LT). The discovery of these attributes of virulence resulted in a better understanding of the pathogenesis of ETEC-induced diarrheal disease. Numerous studies have also demonstrated that vaccination with a virulence factor (i.e.,the purified K99 antigen) can prevent pilus mediated adhesion of ETEC in calves and therefore prevent ETEC-induced diarrheal disease. The mechanisms involved in

protection by pilus antibodies require further research. Among the mechanisms thought to be involved are agglutination, opsonization, bactericidal or bacteriostatic effects or plasmid curing.

OBJECTIVES

The objectives of this research were:

1. To determine if the purified K99 pilus antigen of *E. coli* is suitable for use as an *in utero* vaccine.

2. To evaluate the resistance of K99 pilus-vaccinated calves to exposure to virulent K99 positive ETEC at 4 hours of age.

3. To determine whether K99 pilus-specific antibodies are produced in the serum and intestines of calves following *in utero* vaccination.

4. To determine the effect of *in utero* vaccination at 7 months' gestation on serum and colostral K99 antibody titers in cows at parturition.

5. To measure the distribution of class and subclass K99 pilus-specific antibodies (whether IgG_1, IgG_2, IgM or IgA) in serum and intestines of calves following *in utero* vaccination.

MATERIALS AND METHODS

Experimental Animals and Design

Twelve pregnant Holstein cows were used in this research. Breeding dates were available for most of the cows, and they were verified by rectal palpation at frequent intervals. The cows were maintained on pasture with an open-sided barn for protection from inclement weather. They were fed a recommended hay, concentrate ration supplemented with minerals and vitamins during gestation.

Cows were vaccinated at approximately 7 months' gestation. It was planned to give each cow 10 mg of purified K99 pili or sterile physiological saline, but following abortions it was decided to reduce the dose of pili to 5 mg. Five cows were given 10 mg pili, 3 were given 5 mg pili, 3 were given sterile physiological saline, and 1 was unvaccinated (Tables 1 and 3).

Cows were allowed to proceed through normal parturition, and calves were immediately placed in sterilized, plastic-film isolators. Calves were fed approximately 4 liters of autoclaved (250 F for 35 minutes) whole milk each day.

New Zealand White rabbits were used for the production of K99 and K88 antisera. The rabbits were injected twice at 4-week intervals with purified pili in Freund's complete adjuvant.[a] Multiple subcutaneous

[a]Colorado Serum Co. Laboratories, Denver, CO 80216.

Table 1. Experimental design

12 pregnant Holstein cows

3 saline controls 1 noninoculated control	8 *in utero* vaccinated with purified K99 pili

serum from cows at vaccination

parturition
serum from calves
serum and colostrum from dams

calves maintained in isolators
colostrum deprived

calves challenged at 4 hours with 10^{11} *E. coli* (B44; 09:K30, K99)

necropsy

intestinal secretions	ileum for FA	ileum for bacterial counts	histopathology sections

injection sites were used and approximately 0.1 ml of material was injected at each site. The rabbits were bled 8 to 10 days after the second injection and the serum separated and stored at -70 C.

Vaccine

Purified K99 was prepared by Isaacson as described by Isaacson (1977). Briefly, K99-positive bacteria were grown in 1 liter batches of trypticase soy broth[b] (TSB) in 2 liter Erlenmeyer flasks at 37 C while shaking at 200 rpm for 18 hours. Cells were collected by centrifugation at 17,000 x g for 10 minutes and resuspended in phosphate buffered saline (PBS), pH 7.2. The K99 antigen was extracted at 4 C by homogenization in a Sorvall Omnimixer,[c] set at 4, for 30 minutes. Bacteria were then removed by centrifugation at 17,000 x g for 10 minutes at 4 C, and K99 antigen was reextracted. The 2 supernatants were pooled, precipitated with ammonium sulfate (10.6 g/100 ml) at 4 C, and after 30 minutes the pellet was removed by centrifugation (20,000 x g for 10 minutes) and discarded. Ammonium sulfate (11.3 g/100 ml) was again added to the supernatant. After 30 minutes of stirring, the pellet was removed by centrifugation, resuspended in PBS and dialyzed overnight against PBS. This material was applied to a diethylaminoethyl Sephadex A-50 column.[d] The fractions obtained from the column were assayed for K99 antigen (Isaacson, 1977), and the material appeared to be purified K99 pili. The pili were lyophilized in 10 mg aliquots, stored at -70 C and reconstituted with sterile physiological saline (1 ml/mg pili) immediately prior to use.

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[b] Becton, Dickenson & Co., Cockeysville, MD.

[c] Ivan Sorvall, Inc., Newtown, CT 06470.

[d] Pharmacia Fine Chemicals, Piscataway, NJ.

Vaccination Procedure

The technique used was a nonsurgical method developed by Conner at Michigan State University and described in detail by Olson (1975). The vaccination site was the point at which the fetus was most easily palpated by abdominal ballottement of the right flank. If fetal ballottement was not possible, the point of vaccination was located 5 cm dorsal and 25 to 30 cm anterior to the fold of the right flank. The area was clipped and cleansed with antimicrobial solutions.[e] The skin, muscle layers and peritoneum were locally aneshtetized[f] using sterile disposable syringes and needles. The operator and an assistant wore sterile gloves in order to assure aseptic technique.

A small skin incision was made over the anesthetized area and a 12 gauge 5 cm needle was inserted through muscles and peritoneum. This needle acted as a cannula into which a 16 guage 30 cm needle was inserted and gently pushed through the uterine wall until the tip struck the fetus. Location of the needle tip was determined by aspiration with an attached 10 ml syringe. Amniotic fluid is normally clear, colorless and mucoid. If the needle tip was not within the amniotic cavity, watery, amber-colored allantoic fluid was frequently withdrawn into the syringe. It was then necessary to redirect the needle and reassess its location prior to introduction of the vaccine. Following vaccination, a topical dressing[g] was applied to the skin.

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[e]Betadine Surgical Scrub and Solution, Purdue Frederick Co., Norwalk, CT.

[f]Procaine HCl Solution, 2.5%, Bio-Ceutic Laboratories, Inc., St. Joseph, MO.

[g]Topazone, Eaton Veterinary Laboratories, Norwich, NY.

Amniotic fluid was cultured to determine if bacteria were present at the time of vaccination. All cows were given intramuscular injections of vitamins A and D[h] (5 ml) and selenium-vitamin E[i] (5 ml) at the time of *in utero* inoculation because in previous research, calves had died with lesions of vitamin E-selenium deficiency (Newman, 1978). Selenium-vitamin E[i] injections were repeated where applicable 3 weeks prior to parturition. Blood samples were collected from the cows at the time of vaccination. Serum was separated and stored at -70 C.

Parturition

Cows were allowed to proceed through normal parturition. When parturition was imminent, cows were closely observed and calvings were supervised. All calves were delivered by traction using obstetrical chains and placed on clean plastic sheets. They were immediately transferred to a permanent calf isolator. The isolator system has been described (Mullaney, 1979). Calves were briskly rubbed and dried. Blood samples were collected from the jugular vein, and the serum was separated and stored at -70 C. Blood and colostrum were collected from the cows immediately after parturition. The colostrum was centrifuged at 100,000 x g for 2.5 hours and the clear colostral whey separated and stored at -70 C.

Challenge Inoculum

The *E. coli* strain used for challenge of calves was B44; 09:K30:K99. It was prepared as described by Isaacson et al. (1977b). A K99 piliated

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[h]Vitamin A & D Injectable, Pfizer, Inc., New York, NY.

[i]BO-SE Injection, Burns-Biotec Laboratories,Oakland, CA.

colony was selected from a blood agar plate and inoculated into tubes of TSB. After growth overnight at 37 C, bacteria from the tubes were inoculated into flasks of TSB and incubated for 18 to 20 hours at 37 C. Bacteria were harvested by centrifugation at 7,000 x g for 10 minutes and resuspended in fresh TSB containing 20% sterile glycerol to a concentration of 10^{10} bacteria/ml. Aliquots were stored at -70 C. Each calf received 10 ml (10^{11} ETEC) of this solution. Calves were allowed to nurse 1 liter of autoclaved whole milk at 4 hours of age. Then the challenge inoculum was drawn into a 10 ml syringe, and the calf was orally inoculated. After challenge, the calf was allowed to nurse a second liter of autoclaved whole milk. The calves were closely observed following challenge. All except calf 14 were given the challenge inoculum at 4 hours of age. This calf was premature and appeared very weak after birth. Challenge inoculation was delayed until the calf was 48 hours of age.

Collection of Specimens

Calves which died following challenge inoculation were necropsied as soon as possible after death. Calves which survived were electrocuted 72 hours postchallenge. They were placed in dorsal recumbency for necropsy. Sections of lung, liver, kidney and mesenteric lymph node were collected aseptically for bacteriologic culturing. Five centimeters of terminal ileum (1 meter anterior to the ileocecal valve) and contents were placed in a sterile container and used for bacterial count determinations. Segments of ileum from the same location in each calf were cut into thin slices and placed on cork discs to which a few drops of embedding medium[j] had been added. They were frozen on acetone and dry ice, wrapped

[j] O.C.T. Compound, Lab-Tek Products, Naperville, IL.

in foil, and stored at -70 C. Intestinal secretions were collected in flasks by flushing 300 ml of PBS through 10-foot sections of ileum and jejunum. Tissue sections fixed in 10% buffered formalin included duodenum, jejunum, ileum, colon, abomasum, mesenteric lymph node, liver, kidney, spleen, pancreas, urinary bladder, lung, thyroid, thymus, tongue, trachea, esophagus, and brain.

Bacterial Counts

The number of *E. coli* per 5 cm ileum was determined by a modification of the technique described by Bertschinger et al. (1972). Five centimeter segments of ileum and contents were placed in a jar and the total volume of the sample was adjusted to 30 ml by adding sterile physiological saline. Samples were homogenized in a Sorvall Omnimixer[c] at full speed for 30 seconds. Then 10-fold dilutions were made in sterile saline and dilutions between 10^3 and 10^7 were plated on blood agar and incubated overnight at 37 C. Colonies were identified on the basis of their morphology and counted.

Immunofluorescence Microscopy

An indirect fluorescent antibody (IFA) test was used to detect K99-positive bacteria in the ileum of challenged calves. New Zealand White rabbits were vaccinated subcutaneously with 250 µg of purified K99 pili in Freund's complete adjuvant. Four weeks later they were vaccinated again with a similar dose. One week after the second vaccination, the rabbits were bled and the K99 antiserum separated and stored at -70 C.

Frozen sections of ileum were cut at a thickness of 8 µm on a cryostat.[k] Sections were incubated for 30 minutes at 37 C on glass

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[k]Pearse-Slee Cryostat, Type 'H', Slee Medical Equipment, Ltd., Lanier Works, London SE 13.

slides and fixed in acetone for 10 minutes. Fixed tissues were layered with K99 antiserum (diluted 1:64 in PBS, pH 7.3), incubated for 30 minutes at 37 C, rinsed, and washed in 2 changes of PBS for 30 minutes while stirring gently. Tissues were then stained with the second antibody, which was fluorescein-labeled goat immunoglobulin G prepared against rabbit immunoglobulin G.[1] The second antibody was diluted 1:8 in PBS. Incubation and washing times were the same as for the first antibody. The tissues were mounted in buffered mounting medium (9 parts glyercol, 1 part PBS, pH 7.2) and examined under a fluorescent microscope. For controls, normal rabbit serum and K88 antiserum were used as the first antibody. The K88 antiserum was prepared in the same way as the K99 antiserum using purified K88 pili.

Sections of ileum were examined for rotavirus, and sections of ileum and colon were examined for coronavirus using direct immunofluorescence. The methods used have been described by Mebus et al. (1971b) and Mebus et al. (1973).

Antibody in Intestinal Secretions

Intestinal secretions were centrifuged at 500 x g for 10 minutes to remove large particles and mucoid matter. The supernatant was measured, and any amount in excess of 250 ml was discarded. Ammonium sulfate was added during a 30-minute period to precipitate the immunoglobulins out of solution (31.3 g/100 ml contents). The solution was allowed to stir an additional 60 minutes after the ammonium sulfate was dissolved.

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[1]Research Products, Miles Laboratories, Inc., Elkhart, IN.

The resulting solution was centrifuged at 10,000 rpm for 30 minutes. The supernatant was discarded and the pellet resuspended in 1 ml of 50% saturated ammonium sulfate in PBS, pH 7.4. The volume was increased to 20 ml with ammonium sulfate-PBS and centrifuged for 30 minutes at 10,000 rpm. The wash procedure was repeated twice. The resulting pellet was dissolved in PBS to a final volume of 3 ml. Samples were dialyzed against PBS, pH 7.4, in 2-liter Erlenmeyer flasks on a magnetic stirrer for 3 days at 4 C. The PBS was changed twice daily, and the resulting samples were stored at -70 C.

Immunoelectrophoresis

Methods described by Scheidegger (1955) were used for immunoelectrophoresis. Barbital buffer[m] was prepared as described previously by Leid and Williams (1974). Agar-gel (2%) was prepared by the addition of barbital buffer to dried agar,[n] and the mixture was boiled until all the agar had dissolved. One percent merthiolate was added to the melted agar as a preservative, giving a final concentration of 0.01%. Six microscope slides, cleaned with alcohol, were placed in an electrophoresis frame, and the frame was filled with melted agar. When the agar hardened, wells and troughs were cut. Bovine whole serum or whey was placed in the wells, and electrophoresis was carried out with a current of 10 amperes per frame for 60 minutes. Antiglobulins (anti-bovine IgG_1, IgG_2, IgM and IgA)[o] undiluted or diluted 1:2, 1:4 or 1:8 were placed in the troughs, and the frames were incubated in a moist

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[m]Scientific Products, 17150 Southfield Road, Allen Park, MI.

[n]Noble Agar, Difco Laboratories, Detroit, MI.

[o]Research Products, Miles Laboratories, Inc., Elkhart, IN.

chamber at room temperature for 48 hours. Slides were washed in 2% sodium chloride and distilled water for 2 days with 2 changes of washing solution. Slides were dried and stained[p] for 10 minutes and destained with 2% glacial acetic acid. Stained slides were photographed[q] using black and white film.[r]

Enzyme-Antibody Conjugation

Antibovine immunoglobulin and antibovine IgG_1, IgG_2, IgM and IgA were respectively conjugated to alkaline phosphatase as described by Engvall and Perlmann (1972). Alkaline phosphatase[s] (Type VII enzyme), which is a suspension of 5 mg/ml protein in 3.2 M ammonium sulfate, was used. The source of alkaline phosphatase was calf intestine. Three-tenths milliliter of the enzyme suspension was centrifuged (200 rpm for 10 minutes) at 4 C. The clear supernatant (0.2 ml) was discarded, and the remaining pellet was mixed with 0.1 ml of each antiglobulin solution. Each mixture was dialyzed for 24 hours against PBS,pH 7.3, in 2 liter Erlenmeyer flasks. Then 10 µl of 4.2% glutaraldehyde in PBS were added, yielding a final glutaraldehyde concentration of 0.2%. The reaction was allowed to proceed for 2 hours at room temperature. The mixture was diluted to 1 ml with PBS, dialyzed for 24 hours against PBS and stored at 4 C.

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[p]Aniline Blue Black, Matheson, Coleman and Bell, Norwood, NJ.

[q]Polaroid Mp-3 Land Camera, Polaroid Corp., Cambridge, MA.

[r]Polaroid Type 55 Positive/Negative Land Film, Polaroid Corp., Cambridge, MA.

[s]Sigma Chemical Co., St. Louis, MO.

K99 Antibody Determination

K99 pilus-specific antibody titers in serum, colostrum and intestinal secretions were determined using an enzyme-linked immunosorbent assay (ELISA) (Table 2). Rigid polystyrene microtiter plates[t] were used. Ten µg of purified K99 in 100 µl of PBS (0.01 M sodium phosphate; 0.85% sodium chloride; pH 7.3) were added to each well because previously Isaacson et al. (1980) had determined this was the optimal concentration of antigen. After incubation at 37 C for 2 hours, the plates were maintained overnight at 4 C. The wells were then washed 3 times with PBS (3 minutes per wash) and then were filled with 1% bovine serum albumin[u] in PBS. After incubation for 30 minutes at 37 C, the wells were washed 3 times with PBS-Tween 20[v] which contained 0.05% Tween 20. Then serial 2-fold dilutions of test serum, whey and intestinal secretions were made with PBS-Tween 20 used as diluent. One hundred microliters was added per well, and each dilution was run in duplicate. The following controls were also run daily: 1) normal bovine serum obtained immediately after birth from a calf that had not been vaccinated with pili and had not ingested colostrum was used as a negative control; 2) a serum sample from a cow that had been vaccinated twice with purified K99 and was known to have K99 antibodies[w] served as a positive control. Each day of testing, 20 wells contained normal bovine serum diluted 1:100 in PBS-Tween 20. Serial 2-fold

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[t]Dynatech Laboratories, Inc., 900 Slaters Lane, Alexandria, VA.

[u]Sigma Chemical Co., St. Louis, MO.

[v]E. H. Sargent & Co., 7300 North Linder Avenue, Skokie, IL.

[w]Kindly supplied by Dr. S. Acres, Saskatoon, Canada.

Table 2. Method for detecting K99 pilus-specific antibody using enzyme-linked immunosorbent assay (ELISA)

Layer	Material	Conc./Dilutions	Incubation Temp.	Incubation Time
I	K99	100 µg/ml in PBS, 0.1 ml in each well	37 C 4 C	2 hrs ON*
wash 3X		PBS		3 mins
II	BSA**	1% in PBS	37 C	30 mins
wash 3X		PBS-Tween 20		3 mins
III	a. Test sera	2-fold dil. in PBS-Tween (100 µl/well)	37 C	2 hrs
	b. STD*** sera	2-fold dil. in PBS-Tween (100 µl/well)	37 C	2 hrs
	c. NBS****	1:100 in PBS-Tween (100 µl/well)	37 C	2 hrs
wash 3X		PBS-Tween		3 mins
IV	rabbit anti-bovine conjugate	1:2000 in PBS-Tween	37 C	2 hrs
wash 4X		PBS-Tween		3 mins
V	substrate	1 mg/ml in 0.05 M Na_2CO_3 and 0.001 M $MgCl_2$ (100 µl/well)	R.T.	30 mins
VI	NaOH(.33 N)	150 µl/well	---	---

Read at 400 nm blanked vs distilled H_2O.

* Overnight
** Bovine serum albumin
*** Standard sera, served as positive control
**** Normal bovine sera, served as negative control

dilutions (each in duplicate) of the positive control were also run daily. The positive control also served as a standard to correct for daily variations. For example, during preliminary testing a titer of 2048 was established for the positive control. If the titer obtained during a particular day's testing was 1024, then titers obtained for test material on that day were multiplied by 2. Conversely, if the positive serum yielded a high titer (i.e., 4096), then the titers of the test materials were divided by 2 for that day. After incubation for 2 hours at 37 C the wells were washed 3 times with PBS-Tween 20. Then 100 µl of rabbit anti-bovine serum[x] conjugated to alkaline phosphatase and diluted 1:2000 in PBS-Tween 20 was added to each well. Preliminary testing of conjugate at dilutions of 1:1000, 1:2000 and 1:5000 established 1:2000 as the optimum dilution. After incubation for 2 hours at 37 C the wells were washed 4 times with PBS-Tween 20. Then 100 µl of substrate, p-nitrophenyl phosphate,[y] containing 1 mg/ml in 0.05 M Na_2CO_3: 0.001 M $MgCl_2$, pH 9.8, was added to each well. The time of addition of substrate to each row of wells was recorded using a stopwatch. The reaction proceeded in the dark room at room temperature. After exactly 30 minutes, the reaction was stopped by adding 150 µl of 0.33 N sodium hydroxide to each well. Samples were read at 400 nm on a spectrophotometer[z] fitted with a 200 µl aspirating microcuvette.

Endpoint titers were established as follows. A mean negative control value was obtained and 2 standard deviations from that value was <0.1. The lowest dilution of a test serum, whey or intestinal

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[x]Research products, Miles Laboratories, Inc., Elkhart, IN.

[y]Sigma Chemical Co., St. Louis, MO.

[z]Gilford Stasar II Spectrophotometer, Gilford Instruments, Inc., Oberlin, OH.

secretion having an absorbency at 400 nm of ≥0.1 from the mean negative control value was designated the endpoint titer.

K99 Pilus-Specific Class and Subclass Antibody Determination

K99 pilus-specific IgG_1, IgG_2, IgM and IgA in serum and intestinal secretions were also determined using ELISA. The procedure was the same as described for K99 antibody determination, except that rabbit antibovine IgG_1, IgG_2, IgM and IgA, each conjugated to alkaline phosphatase, were used instead of antibovine serum.

Histopathologic Technique

Tissues fixed in 10% buffered formalin were embedded in paraffin, sectioned and stained with hematoxylin and eosin according to established procedures (Luna, 1968). Sections of ileum were also stained with a Giemsa stain to demonstrate bacteria adherent to villous epithelium.

RESULTS

In utero Vaccination

Control cows maintained normal gestation and calved 36 to 53 days following deposition of sterile physiological saline into the amniotic fluid. Five of eight pilus vaccinated cows either aborted or had premature live calves (Table 3). After 2 cows aborted, the dose of vaccine was reduced from 10 to 5 mg of pili. Three cows were given the reduced dose and 1 aborted.

Response of Calves to Challenge Inoculation

Control calves became depressed and developed diarrhea 10 to 15 hours following oral challenge. Soon after the commencement of diarrhea, the calves stopped drinking autoclaved whole milk from a nursing bottle. Severe depression, recumbency and dehydration were observed prior to death. All control calves died 24 to 48 hours postchallenge (Table 4).

Calves in the vaccinated group (except calf 14) developed essentially similar clinical signs as the control group. Three of four calves died 30 to 40 hours following challenge. Calf 14 remained clinically normal during the postchallenge observation period. This calf excreted semisolid whitish-colored feces and did not at any time excrete fluid feces. Normal appetite was maintained. The calf drank 2 liters of autoclaved whole milk twice each day. The calf was euthanatized 72 hours postchallenge.

Table 3. Interval between *in utero* vaccination and parturition

Groups	Dose	Cow No.	No. of Days	Result
Non-inoculated control	---	5	---	normal calf
Saline controls	10 ml	1	53	normal calf
	10 ml	2	36	normal calf
	10 ml	3	44	normal calf
Vaccinates	10 mg pili in 10 ml saline	4	3	aborted
	5 mg pili	7	41	normal calf
	10 mg pili	8	4	aborted
	5 mg pili	10	53	normal calf
	10 mg pili	11	15	premature live
	5 mg pili	12	6	aborted
	10 mg pili	14	12	premature live
	10 mg pili	17	57	normal calf

Table 4. Response of calves to challenge with K99 positive enterotoxigenic *E. coli*

Groups	Calf No.	Onset of Diarrhea	Outcome
Non-inoculated control	5	15 hours	died 48 hours
Saline controls	1	10-14 hours	died 26 hours
	2	12 hours	died 30 hours
	3	10-12 hours	died 24 hours
Vaccinates	7	12 hours	died 34 hours
	10	10-15 hours	died 40 hours
	14	no diarrhea	clinically normal
	17	12-15 hours	died 30 hours

Gross Lesions

The appearance of the digestive tract in both control and vaccinated calves was similar except for calf 14. The calves had fluid material in the small intestine, and in the large intestine the fluid contents frequently contained gas bubbles. Calf 14 had semisolid contents in both large and small intestines. Petechial hemorrhages were not observed on either the serosal or mucosal surface of the intestine of any calf. All other internal organs appeared normal on macroscopic examination, except for the lungs of calf 2. The apical and cardiac lobes of the lungs of this calf had small patchy dark red areas which appeared to be congested but not consolidated. The conclusions at necropsy were that all calves, except calf 14, had diarrheal disease and associated dehydration which was most severe in calves that survived longest after the onset of diarrhea.

Immunofluorescence

Sections of ileum were examined by the fluorescent antibody (FA) technique for K99, rotavirus and coronavirus. Sections of spiral colon were also examined for coronavirus. All calves challenged with K99 positive ETEC (except calf 14) had layers of K99 positive bacteria adherent to villous epithelium (Table 5). It was unfortunate that in many sections epithelium had sloughed off the villi due to postmortem autolysis. This made interpretation difficult. However, in sections where K99-positive bacteria were observed adherent to the sloughed epithelial cells, the calves were designated positive (Figure 1). In some sections positive fluorescence of individual bacteria was observed (Figure 2). Sections from all calves were FA negative for rotavirus and coronavirus.

Table 5. Results of fluorescent antibody test for K99, rotavirus and coronavirus

Groups	Calf No.	K99	Rotavirus	Coronavirus
Non-inoculated control	5	+	-	-
Saline controls	1	+	-	-
	2	+	-	-
	3	+	-	-
Vaccinates	7	+	-	-
	10	+	-	-
	11[a]	-	-	-
	14	-	-	-
	17	+	-	-

[a]Not challenged because calf died a few hours after birth.

Figure 1. Photomicrograph of a frozen section of ileum from calf 17, stained with anti-K99 and fluorescein-conjugated goat anti-rabbit IgG. Notice a layer of fluorescence adherent to desquamated villous epithelium (400 X).

Figure 2. This photomicrograph demonstrates positive fluorescence of individual bacteria. Immunofluorescent stain (500 X).

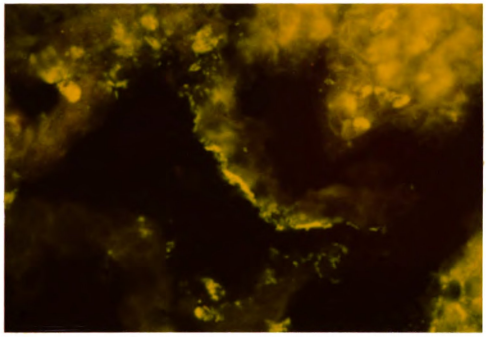

Figure 1

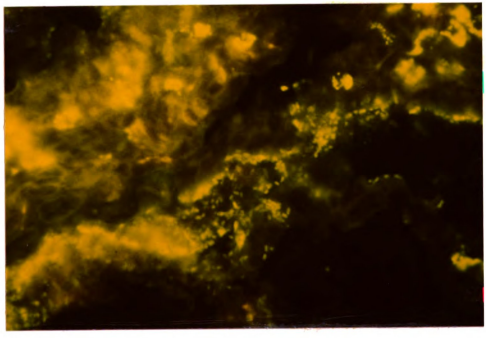

Figure 2

Bacterial Contamination of Internal Organs

Samples of lung, liver, kidney and mesenteric lymph node were cultured from all calves, and a mixed bacterial population was isolated (Table 6). *Escherichia coli* was isolated from these organs in all calves, except calf 14, where no growth was obtained.

Colonization Counts

The number of *E. coli* per 5 cm of ileum in control calves was in each case greater than 10^{10}. This indicated that colonization of the small intestine had occurred. Colonization of the small intestine also occurred in vaccinated calves based on numbers of *E. coli* counted (Table 7), but counts were either 10 or 100 times lower than in control calves. The bacterial count obtained from the ileum of calf 14 was discarded when it was discovered that the saline solution used for dilution was contaminated.

Histopathologic Lesions

Lesions were generally confined to the distal half of the small intestine. Postmortem autolysis made interpretation of intestinal lesions difficult. However, the following lesions were considered to be significant.

Ileum

The ileum of calf 14 appeared histologically normal (Figures 3 and 4). Lesions in control calves varied from villous atrophy in calf 3 to acute neutrophilic ileitis in calf 1. Calf 1 had mild neutrophil infiltration into the lamina propria and large numbers of neutrophils in the lumen located especially on the surface of dome lymphoepithelial cells (Figure 5). Focal necrosis of lymphoepithelial cells had occurred

Table 6. Results of culture of samples of lung, liver, kidney and mesenteric lymph nodes taken at necropsy

Calf No.	Bacteria Isolated	Lung	Liver	Kidney	Mesenteric Lymph Node
5	*E. coli*	L	L	L	L
	Klebsiella pneumoniae	M	M	M	M
1	*E. coli*	M	M	M	M
	Acinetobacter sp.	M	M	M	M
2	*E. coli*	L	L	L	H
	K. pneumoniae	H	H	H	NG
3	*E. coli*	M	M	M	M
7	*E. coli*	L	M	M	H
	Streptococcus sp.	L	NG	NG	NG
10	*E. coli*	L	M	M	H
14	*E. coli*	NG	NG	NG	NG
	K. pneumoniae	L	NG	NG	NG
17	*E. coli*	H	H	H	H

L = light growth
M = medium growth
H = heavy growth
NG = no growth

Table 7. Estimated number of *E. coli*/5 cm of ileum

Groups	Calf No.	Bacterial Count
Non-inoculated control	5	10^{10}
Saline controls	1	10^{10}
	2	10^{10}
	3	10^{10}
Vaccinates	7	7.5×10^{8}
	10	3×10^{8}
	14	---*
	17	5×10^{9}

*Result not included because diluent was contaminated.

Figure 3. Photomicrograph of the ileum of calf 14, which appeared normal histologically. Notice the long slender or bifurcate villi and the apex or dome (D) of lymphoid follicles projecting into the lumen. H&E stain (64X).

Figure 4. Higher magnification of the ileum of calf 14. Notice the uniform, nonvacuolated dome epithelium with intraepithelial lymphoid cells (arrows). Compare with villous epithelium which contains numerous goblet cells. H&E stain (160X).

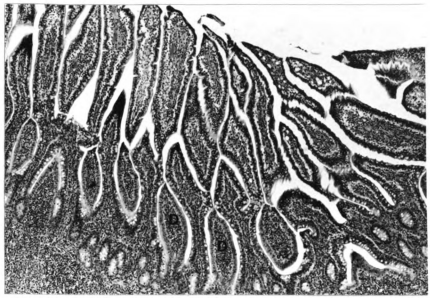

Figure 3

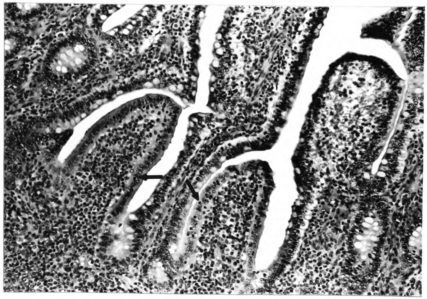

Figure 4

in some areas (Figure 6), exposing the apex of lymphoid follicles to the intestinal lumen. Neutrophils were generally associated with these foci of necrosis. Other control calves had mild hypercellularity of the lamina propria due to neutrophil infiltration.

Peyer's patches in infected control calves appeared depleted of lymphocytes, and in calf 2 occasional lymphoid follicles were completely infiltrated with neutrophils (Figures 7 and 8). Villi were almost completely denuded of epithelium due to postmortem autolysis. In areas where epithelium still covered villi, large numbers of bacteria were observed adherent to villous epithelium (Figure 9).

In vaccinated calves 7, 10 and 17, mild neutrophil infiltration into lamina propria was observed. This was most pronounced in calf 17. It was difficult to find bacteria adherent to villous epithelium in these calves. Peyer's patches appeared depleted of lymphocytes.

Mesenteric Lymph Nodes

All calves, except calf 14, had mild to moderate neutrophil infiltration into lymph nodes. The neutrophils were generally located in the subcapsular sinuses (Figures 10 and 11) and occasionally were also observed in medullary sinuses.

Other Organs

Mild neutrophilic bronchopneumonia was observed in calf 2. The neutrophils were located in bronchioles and surrounding alveoli. Other calves had diffuse congestion in the lungs without any cellular infiltration. Calves 7 and 17 had centrilobular congestion in the liver, and hepatocytes appeared pyknotic in some areas around the central veins.

Figure 5. Photomicrograph of the ileum of calf 1. Notice the large numbers of neutrophils on the luminal surface of the dome lymphoepithelial cells. H&E stain (160X).

Figure 6. Higher magnification of Figure 5. Notice necrosis of dome lymphoepithelial cells with neutrophil infiltration. This necrosis has exposed the apex of a lymphoid follicle to the intestinal lumen. H&E stain (400X).

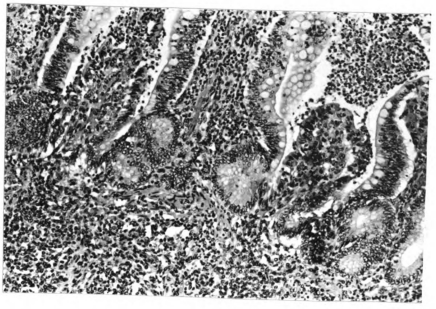

Figure 5

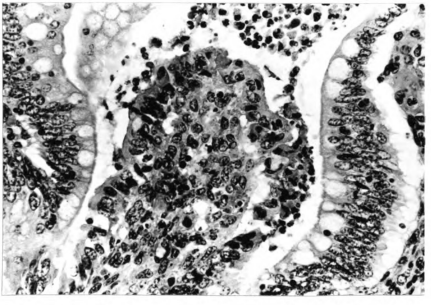

Figure 6

Figure 7. Photomicrograph of a Peyer's patch in the ileum of calf 2. Notice the lymphoid follicle in the center is completely infiltrated with neutrophils. H&E stain (64X).

Figure 8. Higher magnification of Figure 7 to illustrate total replacement of a lymphoid follicle with neutrophils. H&E stain (160X).

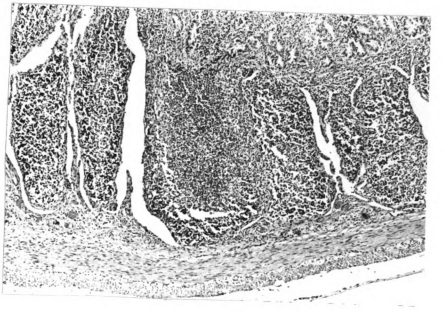

Figure 7

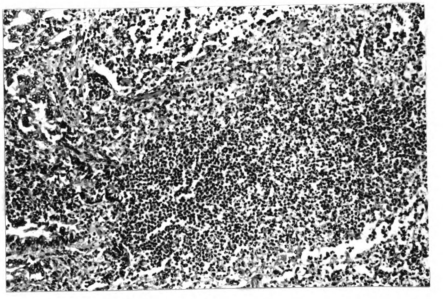

Figure 8

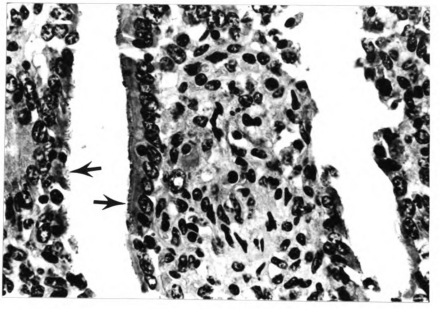

Figure 9. Photomicrograph of the ileum of calf 5. Notice the layer of bacteria adherent to villous epithelium (arrows). H&E stain (640 X).

Figure 10. Photomicrograph of a mesenteric lymph node of calf 10. Notice the neutrophils infiltrated into the subcapsular sinus (arrow). H&E stain (160X).

Figure 11. Higher magnification of Figure 10 illustrates neutrophils in subcapsular sinus of a mesenteric lymph node. H&E stain (400X).

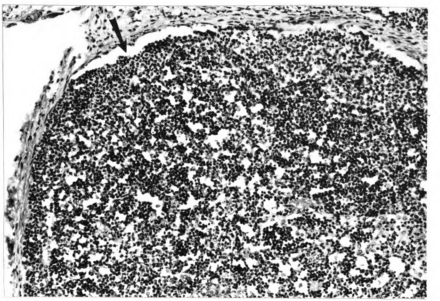

Figure 10

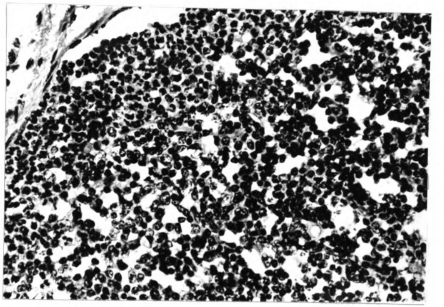

Figure 11

K99 Pilus-Specific Antibody Titers

K99 pilus-specific antibody titers in sera from cows at the time of vaccination varied between 0 and 32 (Table 8). At the time of calving titers varied between 0 and 64. *In utero* vaccination therefore had little effect on K99 antibody in cow sera. There was also little variation in colostral K99 antibody titers between control and vaccinated cows. K99 antibody titers in serum from control calves were 0, while in vaccinated calf sera titers were increased to between 16 and 256. Low titers ($\leq$4) were present in intestinal secretions from 2 of 4 control calves, while vaccinated calf titers had increased to between 8 and 256. The 0 titer in the intestinal secretions of calf 12 was not used in the previous comparison, because this calf aborted only 3 days following *in utero* vaccination.

K99 Pilus-Specific IgG_1, IgG_2, IgM and IgA Titers

The anti-bovine IgG_1, IgG_2, IgM and IgA were each checked for monospecificity using immunoelectrophoresis. Each isotype was reacted with whole bovine serum, and anti-IgA was also reacted with bovine saliva. Single precipitin lines were produced with each antiglobulin (Figures 12 through 15).

Immunoglobulin G_1 titers were 0 in control calf sera and increased to between 4 and 256 in vaccinated calves (Table 9). Intestinal secretions from control calves had titers between 0 and 8, while titers in vaccinated calves were increased and varied between 4 and 128.

Immunoglobulin G_2 titers were 0 in sera from control calves and only slightly increased in sera from vaccinated calves (Table 10). Titers in intestinal secretions varied between 0 and 8 in control calves and between 0 and 32 in vaccinated calves.

Table 8. K99 pilus-specific antibody titers

Groups	Cow-Calf No.	Serum: Cow at Vaccination	Serum: Cow at Calving	Serum: Calf at Birth	Colostrum	Intestinal Secretions
Non-inoculated control	5	---	8	0*	0	0
Saline controls	1	0	0	0	8	4
	2	0	0	0	16	4
	3	0	4	0	0	0
Vaccinates	4	32	ND[a]	ND	ND	ND
	7	8	4	64	4	8
	8	0	ND	ND	ND	ND
	10	4	0	128	32	64
	11	0	8	128	16	32
	12	0	0	16	0	0
	14	0	64	256	ND	256
	17	0	8	256	64	16

[a]ND = not determined.

*Titer was negative at a 1:2 dilution.

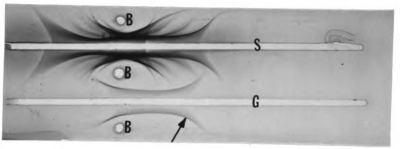

Figure 12. Photograph of the immunoelectrophoretic pattern of anti-bovine IgG_1. Whole bovine serum (B), anti-whole bovine serum (S), anti-bovine IgG_1 (G). Notice the single immunoprecipitate band (arrow) corresponding to the electrophoretic mobility of IgG_1. Analine blue black stain.

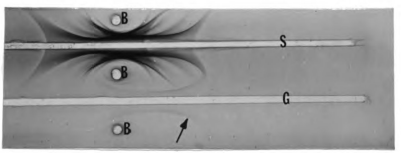

Figure 13. Immunoelectrophoretic pattern of anti-bovine IgG_2. WBS (B), AWBS (S), anti-bovine IgG_2 (G). Notice the single immunoprecipitate band (arrow) corresponding to the electrophoretic mobility of IgG_2

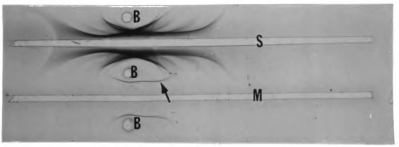

Figure 14. Immunoelectrophoretic pattern of anti-bovine IgM. WBS (B), AWBS (S), anti-bovine IgM (M). Notice the single immunoprecipitate band (arrow) corresponding to the electrophoretic mobility of IgM.

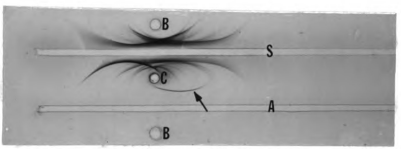

Figure 15. Immunoelectrophoretic pattern of anti-bovine IgA. WBS (B), AWBS (S), bovine colostrum (C), anti-bovine IgA (A). Notice the single immunoprecipitate band (arrow) corresponding to the electrophoretic mobility of IgA.

Immunoglobulin M titers were 0 in sera of all calves except vaccinated calves 12 and 14, where titers were 16 and 32, respectively (Table 11). Titers in intestinal secretions varied between 0 and 8 in control calves and between 0 and 32 in vaccinated calves.

Immunoglobulin A titers were 0 in sera of control calves and varied between 0 and 32 in sera of vaccinated calves. Control calf 1 had an IgA titer of 8 in intestinal secretions, while other controls were negative (Table 12) . Titers in vaccinated calves were increased and varied between 4 and 256.

Overall, IgG_1 predominated in serum from vaccinated calves, while IgA was the predominant immunoglobulin in intestinal secretions. Titers of IgG_2 and IgM were unaltered or only slightly increased in serum and intestinal secretions as a result of pilus vaccination.

Table 9. K99 pilus-specific IgG_1 antibody titers

Groups	Calf No.	Serum	Intestinal Secretions
Non-inoculated control	5	0	8
Saline controls	1	0	4
	2	0	4
	3	0	0
Vaccinates	4	ND[a]	ND
	7	128	16
	8	ND	ND
	10	128	16
	11	256	128
	12	4	4
	14	256	64
	17	256	32

[a]ND = not determined.

Table 10. K99 pilus-specific IgG_2 antibody titers

Groups	Calf No.	Serum	Intestinal Secretions
Non-inoculated control	5	0	2
Saline controls	1	0	8
	2	0	0
	3	0	0
Vaccinates	4	ND[a]	ND
	7	4	8
	8	ND	ND
	10	16	0
	11	8	0
	12	0	16
	14	16	32
	17	16	16

[a]ND = not determined.

Table 11. K99 pilus-specific IgM antibody titers

Groups	Calf No.	Serum	Intestinal Secretions
Non-inoculated control	5	0	0
Saline controls	1	0	8
	2	0	0
	3	0	2
Vaccinates	4	ND[a]	ND
	7	0	4
	8	ND	ND
	10	0	0
	11	0	8
	12	16	32
	14	32	32
	17	0	8

[a]ND = not determined.

Table 12. K99 pilus-specific IgA antibody titers

Groups	Calf No.	Serum	Intestinal Secretions
Non-inoculated control	5	0	0
Saline controls	1	0	8
	2	0	0
	3	0	0
Vaccinates	4	ND[a]	ND
	7	0	128
	8	ND	ND
	10	16	128
	11	32	32
	12	0	4
	14	8	256
	17	0	256

[a]ND = not determined.

DISCUSSION

In utero Vaccination

Calves vaccinated *in utero* with purified K99 pili were either aborted (3 of 8) or were born prematurely (2 of 8). Reduction in the dose of pili in the vaccine from 10 to 5 mg did not appear to influence the outcome, because 1 of 3 cows aborted following reduction of the vaccine dose. It was anticipated that a purified, almost endotoxin free, vaccine would be suitable for use *in utero*. A limulus assay[aa] of the vaccine indicated approximately 1 μg of endotoxin was present per mg of pili. It was considered unlikely that such a small amount of endotoxin would cause abortion, although it was suggested previously (Conner et al., 1973; Gay, 1975) that endotoxin in whole-cell *E. coli* vaccines may have precipitated abortions following *in utero* vaccination.

The non-surgical vaccination procedure used in this research was considered not to be a factor in the occurrence of abortions. Control cows in this and other research (Newman et al., 1978) continued normal gestation following introduction of sterile physiological saline into the amniotic fluid. Factors which precipitated the abortions or premature births were not identified.

[aa]Assayed at the laboratory of Dr. R. J. Moon, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824.

The problem of abortions and premature births following *in utero* vaccination has also been encountered by other researchers (Gay, 1975; Conner et al., 1977; Hamid et al., 1977; Newman et al., 1978). Newman et al. (1978) considered increased fetal corticosteroid levels a possible mechanism of induction of abortion or premature birth following intra-amniotic vaccination with an attenuated coronavirus. However, there appears to be no available evidence to support or contradict that observation because there are no reports in the literature of measurement of corticosteroid levels following *in utero* vaccination. Abortions and premature birth were not a problem when Olson and Waxler (1976) and Wamukoya and Conner (1976) used an *E. coli* bacterin as an *in utero* vaccine. In spite of these conflicting observations, most of the evidence indicates that vaccines used *in utero* against the common enteric pathogens encountered by the neonatal calf,such as ETEC, rotavirus and coronavirus, may adversely affect gestation.

The occurrence of abortions, together with the invasive nature of this technique, lead me to the conclusion that this method is not a feasible one in the prevention of ETEC induced diarrheal disease in calves at the present time. Future research on purified pilus vaccines of *E. coli* should therefore concentrate on using them parenterally in pregnant cows and sows and on evaluating the mechanisms by which pilus-specific antibodies exert their protective effect. The possibility of using purified pili as oral vaccines in the prevention of diarrhea caused by *E. coli* in 3-week-old piglets and also in prevention of *E. coli* induced diarrhea of pigs after weaning are other areas which require future research.

At the start of this experiment, no data were available on the amount of purified K99 pili required for stimulation of the bovine fetal

immune response. Acres et al. (1978) had given purified K99 pili as a subcutaneous vaccine to pregnant cows. They found that 10 mg of pili given twice at an interval of 3 weeks late in gestation had stimulated production of high K99 antibody titers in colostrum. Colostrum-fed calves were protected from fatal diarrhea caused by ETEC following this vaccination procedure. Based on this information, a dose of 10 mg was used, but when abortions occurred the dose was reduced to 5 mg.

Cows were vaccinated *in utero* at approximately 7 months' gestation. It was thought that vaccination at 7 months would reduce the risk of abortions or premature births. This idea was based on results in our earlier research (Newman et al., 1978), where *in utero* vaccination with an attenuated coronavirus in the last month of gestation resulted in abortions or premature births, whereas Mebus and Wyatt (1978) found that introduction of rotavirus into the amniotic fluid of the bovine fetus at 7 months did not adversely affect gestation.

Challenge Inoculation

Each calf in this experiment was orally challenged with 10^{11} K99 positive ETEC. This dose was used becasue in an earlier study (Acres et al., 1979), a similar dose consistently induced fatal enterotoxic colibacillosis in colostrum-fed calves. In the present experiment, enterotoxic colibacillosis occurred in all except 1 calf following challenge. This diagnosis was based on the high bacterial counts obtained from the small intestine and because the FA test was positive for K99. However, there is the possibility that septicemic colibacillosis also contributed to the death of these calves. Moon (1974) noted that almost any strain of *E. coli* is capable of causing septicemia in colostrum-deprived calves. The clinical signs of depression, recumbency

and rapid death in these calves were similar to the clinical signs of septicemic colibacillosis described by Gay (1965). Also, *E. coli* were isolated from samples of lung, liver, kidney and mesenteric lymph node of almost all calves. These isolates may be indicative of septicemia, but there is also the possibility that the *E. coli* invaded the extra-intestinal tissues post mortem because usually 1 hour elapsed between the time of death and the collection of specimens at necropsy. Lesions of septicemia, such as petechial hemorrhages on internal organs, were not observed in any calf during gross examination of organs at necropsy. It would also have been interesting to know if the *E. coli* isolated from the internal organs were of the same serotype as the challenge strain, but methods for serotyping these isolates were not available.

Calf 14 remained clinically normal following challenge. This calf was extremely weak and recumbent following premature birth and was not challenge-inoculated until 48 hours old. The immunity of this calf may have been due to pilus vaccination but could also have been due to age resistance to ETEC, which apparently develops in calves 2 to 3 days after birth (Smith and Halls, 1967a; Runnels et al., 1980).

The dose of challenge organism may have overwhelmed the immune system of the calves in this experiment. Based on the bacterial counts obtained from the ileum of control calves, the dose of ETEC used may have approached LD_{100} (Moon, 1981). Another complicating factor was the isolation of potential pathogens such as *Klebsiella pneumoniae* from the internal organs of these calves. Although they may not have contributed significantly to death of calves in this experiment (clinical signs, colonization counts, and positive fluorescence for K99 all suggested enterotoxic colibacillosis or a combination of enterotoxic colibacillosis and septicemic colibacillosis were the cause of death),

future research involving colostrum-deprived calves could be monitored better by using a completely germfree system.

Immunofluorescence for Detection of K99

An indirect fluorescent antibody test was used in this research to detect K99-positive *E. coli* in the small intestine. This test appears to be quite useful in the diagnosis of ETEC-induced diarrheal disease in calves. However, it is important that ileal sections for immunofluorescent staining be taken immediately after death of calves. In this research, sections were preserved for the IFA test about 1 hour after death of calves. During this time postmortem autolysis caused widespread desquamation of villous epithelial cells in the small intestine. This made interpretation of the test difficult. A positive diagnosis was based on observation of K99 positive bacteria adherent to clumps of desquamated epithelial cells in the lumen of the intestine rather than the observation of fluorescing layers adherent to intact villi (Moon et al., 1978b; Isaacson et al., 1978a). Postmortem autolysis in the small intestine proceeds more rapidly in calves with ETEC-induced diarrheal disease than in non-infected calves (Pearson and Logan, 1978). Denudation of the tips of villi can occur within minutes of death in ETEC-infected calves. Therefore, for practical purposes, calves should be submitted live for diagnosis when ETEC-induced diarrheal disease is suspected.

The FA test for K99 not only detects K99, which is one virulence factor on EPEC in calves, but can also be used as a semiquantitative determination of the number of *E. coli* in the small intestine. In addition, the test determines the location of *E. coli* in the small intestine

(i.e., whether adherent to villi or randomly distributed in the lumen) (Isaacson et al., 1978a).

Another important factor has emerged in the past few years. When K99 is detected by immunofluorescence, it can also be assumed that the K99-positive bacteria are enterotoxigenic (Isaacson et al., 1978a). Initial studies indicated that between 25 and 95% of ETEC isolated from calves were K99 positive (Morin et al., 1976; Moon et al., 1976; Myers and Guinée, 1976). This variation was later demonstrated to be due to difficulties in detection of K99 on freshly isolated *E. coli* strains. Improved detection techniques now indicate that K99 is almost always accompanied by ST production (Isaacson et al., 1978a).

The FA test for K99 is a rapid, simple and inexpensive test. One of the drawbacks associated with the test is that calves must be sacrificed for diagnosis. Nevertheless, according to Isaacson et al. (1978a) this procedure "is most suited to diagnosis of ETEC-mediated diarrhea in calves."

Histopathologic Lesions

The lesions associated with ETEC-induced diarrheal disease were variable in these calves and were not of diagnostic significance. Variable lesions were also reported by Moon et al. (1970) following experimental reproduction of enteric colibacillosis in pigs. Neutrophils, when observed, were most numerous on the luminal surface of the dome of lymphoid follicles. The morphology of the lymphoid follicles in the ileum of the newborn calf was described by Torres-Medina (1981). Probably many calves exude neutrophils into the lumen of the ileum when infected with ETEC, but they are frequently washed away during fixation and processing. This factor may be one explanation for the variation

in neutrophil response observed in this and other experiments. Although the histopathologic lesions of ETEC-induced diarrhea in calves are not diagnostic, the intestines of calves with diarrhea should always be examined histologically, when available, to help diagnose other causes of diarrhea and mixed infections.

Colonization Counts

Bacterial counts indicated that colonization of the small intestine by *E. coli* occurred in both control and vaccinated calves. It is generally assumed that colonization of the small intestine has occurred or that *E. coli* are present in sufficient numbers to cause diarrhea when $\geq 10^8$ *E. coli* are present per 5 cm of ileum (Moon et al., 1978). Vaccinated calves in this experiment had between 10 and 100 times less bacteria per 5 cm of ileum than control calves. However, the validity of these counts may be questionable, because bacterial growth undoubtedly occurred between death of calves and collection of samples.

K99 Antibody Titers

Pilus-specific antibody titers following vaccination were increased in calf serum and intestinal secretions. These titers were quite low when compared to colostral K99 antibody titers following vaccination of pregnant cows with purified pili (Acres et al., 1979). There are a number of possible explanations to account for the low K99 antibody titers in this experiment. The pili, because of their adhesive properties, may have adhered to amniotic membranes when injected. If such adherence occurred, only a portion of the vaccine dose would have been ingested by the calves. Also, pili that were ingested by calves may have adhered to specific receptors on the villous epithelium of the small

intestine. This specific interaction could inhibit (or enhance?) pilus uptake by the gut-associated lymphoid tissue (Peyer's patches) and subsequent activation of the immune system. Also, the ability of orally administered dead bacterial antigens to stimulate the immune system must be considered. Pili used in this experiment resulted in a small increase in antibody titers of vaccinated calves. Moon (1981) found no increase in pilus-specific antibody titers after oral vaccination of gilts with dead piliated strains of *E. coli*. In contrast, other reports demonstrated that dead bacterial antigens given orally *in utero* were effective in protecting newborn calves from challenge exposure with virulent homologous strains (Gay, 1975; Conner et al.,1973). These contrasting reports support the suggestion by Moon (1981) that further studies on the location of bacterial antigens following oral vaccination are necessary to enhance our understanding of immunity to oral vaccines.

Pilus-specific class and subclass antibody titers indicated IgG to be the predominant isotype in vaccinated calf sera and IgA in intestinal secretions. The predominance of IgA in intestinal secretions was expected because IgA is usually the major immunoglobulin associated with protection of mucous epithelium (Porter, 1973b). The predominance of IgG_1 in serum was not expected. Immunoglobulin G_1 usually predominates in calf serum but this is associated with selective concentration of this isotype in bovine colostrum (Porter, 1973b), whereas adult bovine serum usually contains almost equal amounts of IgG_1 and IgG_2.

The ELISA has been adapted in the past few years for quantitating the class or subclass of a specific antibody. Sloan and Butler (1978) reported that cross reactivity occurs between IgG_1 and IgG_2 resulting in higher than expected IgG_2 levels, when only a precipitin assay is

used as a measure of specificity for evaluating antiglobulins. Whether such an elevation of IgG_2 levels occurred in this research was not determined, but the titers obtained for IgG_2 in both serum and intestinal secretions were extremely low.

Different conjugates were used in this research for measurement of each antibody isotype. This means that an outside variable has been introduced when comparative measurements of isotypes are made. To facilitate comparative quantitation when measurement of the distribution of classes and subclasses of a specific antibody, Butler et al. (1980) developed an amplified ELISA. This technique utilizes only one conjugate for measurement of all 4 isotypes and therefore eliminates variations which result from the use of a number of conjugates.

Low titers (≤ 8) of K99 antibodies were quantitated in intestinal secretions of some control calves. These titers may have represented the onset of antibody production by calves as a result of infection with K99 positive ETEC. This explanation seems feasible in view of the findings of Logan and Penhale (1972) where immunoglobulins were detected in the feces of colostrum-deprived calves within 48 hours after infection of calves with EPEC.

SUMMARY

The response of the bovine fetus to intrauterine (oral) inoculation with purified K99 pili of *Escherichia coli* was determined by inoculating 12 cows at approximately 7 months of gestation with purified pili or sterile physiological saline. At birth calves were colostrum-deprived, placed in plastic-film isolators and fed autoclaved whole milk.

Control cows maintained normal pregnancies while 5 of 8 vaccinated cows aborted or had premature live calves. Control and vaccinated calves were orally challenged at 4 hours of age with 10^{11} K99 positive enterotoxigenic *E. coli* (ETEC) and all except 1 vaccinated calf died 24 to 48 hours following challenge. Immunofluorescent examination of ileum for K99 was positive in calves that died following challenge.

K99 pilus-specific antibody values measured by enzyme-linked immunosorbent assay (ELISA) were increased after vaccination with pili in sera from calves at birth and in intestinal secretions from calves at necropsy. Vaccination did not affect antibody values in sera from cows at parturition in comparison to antibody values from cows at vaccination. Antibody values were not increased in colostrum from vaccinated cows. Pilus-specific IgG_1 predominated in sera from vaccinated calves, while IgA was the predominant antibody in intestinal secretions.

These results indicate the ability of the prenatal calf to respond to oral vaccination with purified K99 pili by pilus-specific antibody production in sera and intestinal secretions. Vaccination with pili, however, did not prevent fatal diarrhea caused by ETEC. Abortions and premature birth of calves following vaccination preclude widespread use of this technique.

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