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# Enterotoxigenic *Escherichia coli* Infections in Newborn Calves: A Review

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## ABSTRACT

Diarrhea caused by enterotoxigenic *Escherichia coli* is an infectious bacterial disease of calves that occurs during the first few days of life. The *Escherichia coli* that cause the disease possess special attributes of virulence that allow them to colonize the small intestine and produce an enterotoxin that causes hypersecretion of fluid into the intestinal lumen. These enterotoxigenic *Escherichia coli* are shed into the environment by infected animals in the herd and are ingested by newborn calves soon after birth. There is some natural immunity to enterotoxigenic *Escherichia coli*; however, it often fails to protect calves born and raised under modern husbandry conditions. Hence, methods have been developed to stimulate protective immunity by vaccination of the dam. The protective antibodies are transferred passively to calves through the colostrum.

## INTRODUCTION

*Escherichia coli* cause two common diseases of newborn calves: coli-septicemia in which the bacteria invade the systemic circulation and internal organs (136), and enteric colibacillosis in which the bacteria are localized to the lumen and mucosal surface of the small intestine (71, 139). The pathogenesis of these two diseases is markedly different, and the types of *E. coli* that cause them also differ. Each type possesses unique attributes of virulence that differentiate it from each other as well as from the non-pathogenic group of *E. coli* that are part of the normal digestive flora of healthy calves (136, 137, 139, 140). Hence, those *E. coli* that cause septicemia survive and multiply in the blood and

internal organs of calves (136, 137). Conversely, those that cause diarrhea are equipped to survive locally in the gastrointestinal tract; however, except in the agonal stages of disease, they usually do not penetrate the body beyond the mesenteric lymph nodes nor invade the systemic circulation (139). This paper will concentrate on enterotoxigenic *E. coli* (ETEC), which cause the localized intestinal disease.

## CHARACTERIZATION OF ENTEROTOXIGENIC *Escherichia coli*

To cause diarrhea, ETEC must possess certain specific characteristics or attributes of virulence that play an essential role in the pathogenesis of enteric disease (Figure 1). As their name suggests, the first of these is the ability to produce enterotoxins which are the biochemical mediators of fluid secretion (37, 80, 133, 134). The ETEC can produce two types of enterotoxin, heat labile (LT) and heat stable (ST) (48, 133, 134). The LT is a protein of large molecular weight, similar in structure and function to cholera toxin, and it is produced by ETEC isolated from humans and pigs but not by those isolated from calves (48). In contrast, ST is a small molecular weight protein (121, 133, 134) of which there are two subtypes, STa and STb (13). To date all ETEC isolated from calves produced STa but not STb (13, 30, 128, 129, 140).

The other essential attributes of virulence are structural components of bacterial cells that enable them to survive and multiply in the small intestine (138) (Figure 1). The most important are pili, or fimbriae, which allow the bacteria to attach to epithelial cells and thereby colonize the small intestine (138, 140). Capsular (K) and somatic (O) or bacterial cell wall antigens also play a role in colonization, but their contribution is not as well-defined (16, 49, 50, 138). In addition, flagellar (H) antigens occur on some strains of *E. coli*; however, they are uncommon on calf ETEC, and it is not

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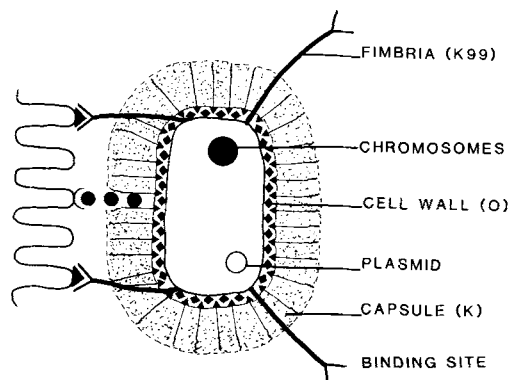


Figure 1. Schematic representation of enterotoxigenic *Escherichia coli* attached to a villous epithelial cell in the small intestine. Attachment is initiated when binding sites (<) on fimbrial antigens (eg. K99) bind to specific receptors (▶) on the epithelial cell surface. Some strains of ETEC also possess capsular (K) antigens which reinforce the primary attachment and provide protection against physiological and immunological defense mechanisms. Enterotoxin released by the bacteria (●) also binds to receptor sites on the epithelial cell (◊). The production of fimbriae and enterotoxin is controlled by genes carried by transmissible plasmids (◊). Both factors are required for enteropathogenicity.

known whether they contribute to virulence. Each of these structural antigens can be identified and typed by serological techniques (104). The various antigens within each group are designated by number, and when all antigens are known for a particular strain, they comprise the serotype of that strain. The classical serotyping system originally included only the O, K, and H antigens. Although pili have been known to occur on *E. coli* for many years (31), they were not incorporated into the system until after their role in pathogenesis of diarrhea was demonstrated. For example, ETEC that have the serotype 09:K30:K99:H – commonly are isolated from diarrheic calves. Some characteristics of these various bacterial antigens are outlined below, and serotypes of ETEC most frequently isolated from diarrheic calves appear in Table 1.

#### Pilus or Fimbrial Antigens

Pili (= hair) or fimbriae (= fringe) are fine proteinaceous filaments that cover the bacterial cell surface. On electron micrographs they appear as a fuzzy coat surrounding the bacteria

(Figure 2). It is becoming clear from the results of recent studies that the ETEC isolated from each animal species possess families or groups of different fimbriae that are important in the pathogenesis of disease in that species (6, 23, 92, 103, 109). Fimbriae on ETEC act as adhesins that attach the bacteria to intestinal epithelial cells (19, 60, 103, 138, 140). Attachment occurs when binding sites on the fimbriae react with specific receptor sites on the epithelial cell surface (Figures 1 and 3). This attachment allows the bacteria to overcome removal by peristalsis and is an essential step in colonization of the small intestine. The most important fimbria on calf ETEC is the K99 antigen, which occurs on most, if not all, isolates known to be virulent for newborn calves (Table 1) (46, 78, 109, 128). When originally discovered, K99 behaved like a capsular antigen in serological tests and inadvertently was assigned a K designation (105). However, it should not be confused with the capsular (K) antigens described below. To detect K99 in vitro, ETEC must be cultured on special media that promote expression of the fimbria (26, 28, 40, 46, 47, 57, 59).

In addition to K99, several other fimbriae, designated as F41 (27, 88, 89, 92), F(Y), F92b (19), Att25 (109), and F210 (17) have been identified on calf ETEC. Of these, F41 is the best characterized. It mediates attachment to calf intestinal epithelial cells in vitro and to the small intestine of newborn lambs (88, 89, 90). This is presumptive evidence that F41 is also an attachment factor for calves in vivo. To date F41 has been found in combination with K99 antigen on calf ETEC belonging to O serogroups 9 and 101 but not on those in other serogroups (88, 89, 92) (Table 1). Strains of K99<sup>-</sup>, F41<sup>+</sup> ETEC have not been isolated yet from calves (J. A. Morris, personal communication); however, they occur in pigs (92). The Att 25 fimbria also has been found in combination with K99 on about 12% of calf ETEC, and K99<sup>-</sup> Att 25<sup>+</sup> strains have been isolated (109, 110). Although ETEC that possess F(Y), F92 b, Att25, and F210 attach to isolated intestinal epithelial cells in vitro or colonize the small intestine of colostrum-deprived calves, conclusive evidence for their role in the pathogenesis of natural cases of diarrhea is still lacking. It is likely that type 1 fimbriae also occur on calf ETEC, and although type 1 mediates attachment to intestinal epithelial cells in vitro, it is not known

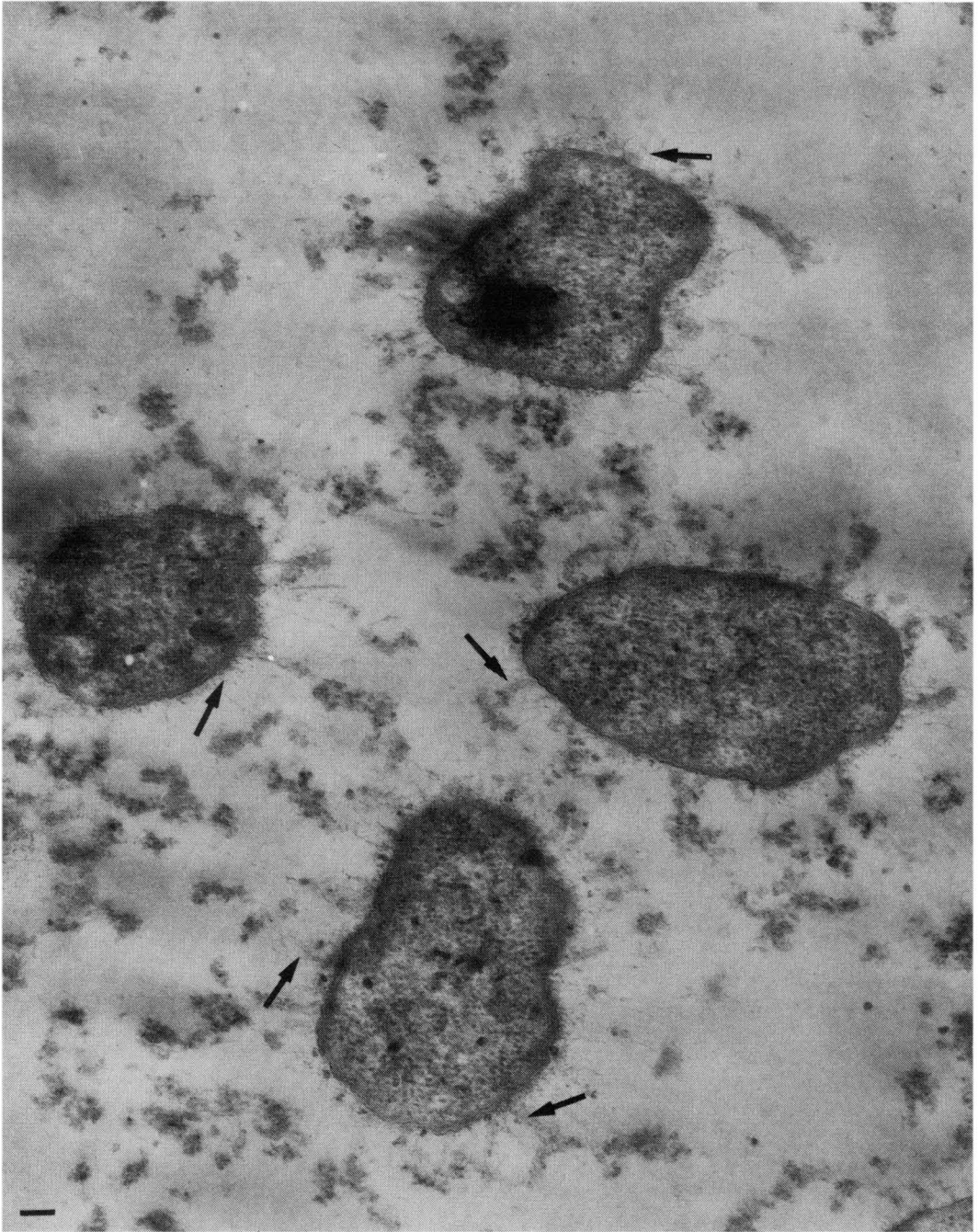


Figure 2. Electron micrograph of enterotoxigenic *Escherichia coli* B44 (serotype O9:K30:K99:F41:H-). Fine K99 fimbriae (→) can be seen as a fuzzy layer extending from the surface of the bacterial cells. The bacteria were treated for 1 h in K99 antibody to stabilize the fimbriae prior to routine fixation. The fimbriae possess binding sites that enable the bacteria to attach to specific receptor sites on the intestinal epithelial cells. (Bar = .1  $\mu$ ).

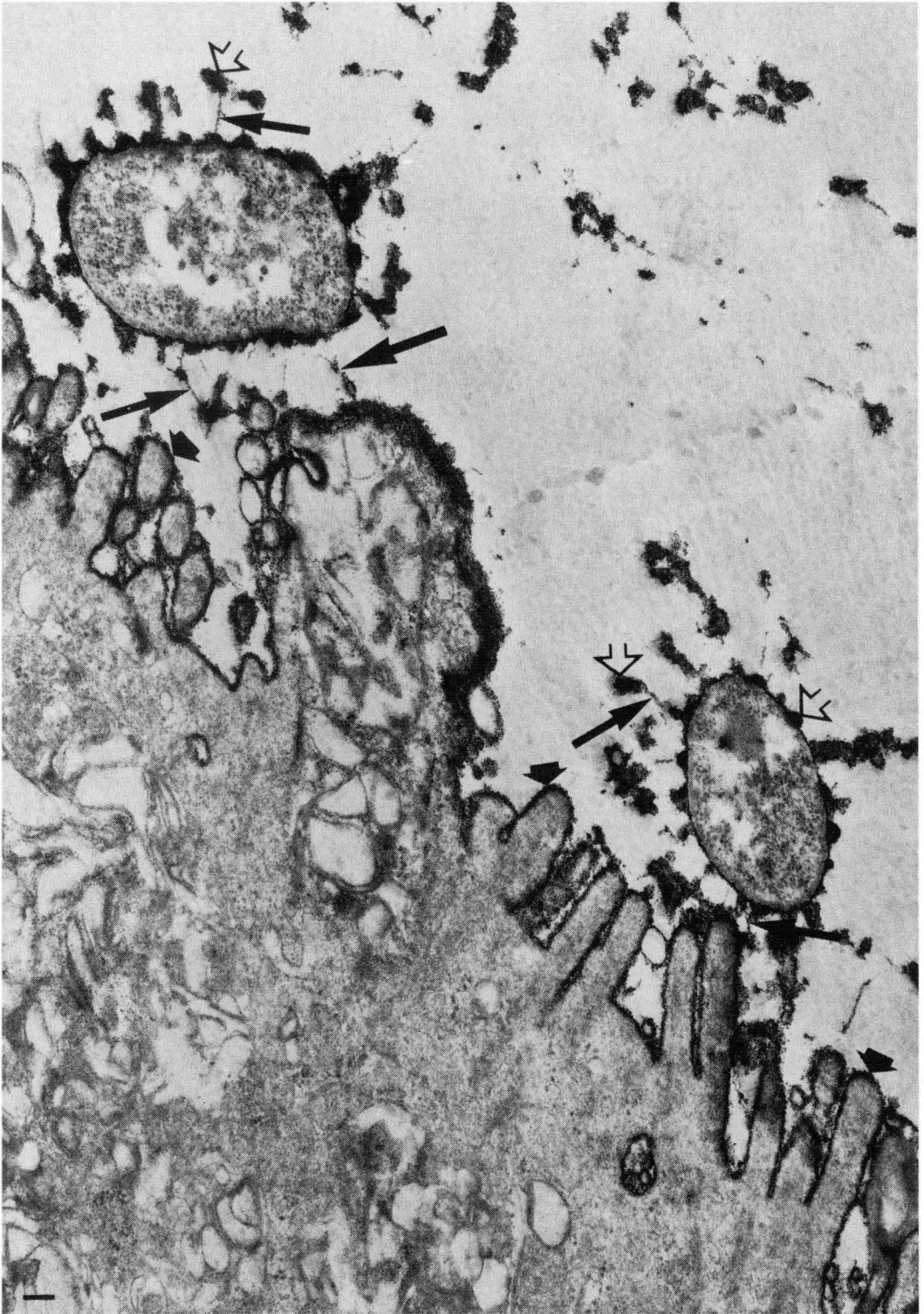


TABLE 1. Serotypes of enterotoxigenic (STa) *Escherichia coli* commonly isolated from diarrheic calves.

Classical antigen			Fimbrial antigen		Type strain	Reference <sup>a</sup>
O	K	H	K99	F41		
8	25	NE <sup>b</sup>	+	-	559	46, 96
8	85	-	+	-	B117	46, 66, 88, 96, 105
8	(A) <sup>c</sup>	27	+	NE	-	unpub <sup>d</sup>
9	30	-	+	+	B44	46, 66, 88, 105
9	35	-	+	+	B42	46, 66, 88, 96, 105
9	55	NE	+	NE	-	66
9	(A)	NE	+	NE	-	21, 46, 66, 113
9	-	-	+	+	B85	88, 105
20	17(?)	NE	+	-	B80	88, 66
20	X106	4	+	NE	-	unpub <sup>d</sup>
20	(A)	NE	+	NE	-	46, 66, 96
26	60	NE	+	NE	-	21, 66
101	28	NE	+	NE	-	46, 96, 113
101	30	NE	+	+	RVC1616	21, 46, 66, 88, 96, 113
101	32	-	+	+	B79	88, 105
101	41	NE	+	NE	-	113
101	(A)	NE	+	NE	-	46, 66, 113
101	-	-	+	+	B41, B111	88, 105
141	85	NE	+	NE	-	109, 122, 128

<sup>a</sup>Enterotoxin producing strains in serogroups 0101:K44:K99, 08:K43:K99 (113), 0138:K81, 014:K+(85), 02 (64), 017 (122, 130), 065, 0124, (122), 0150, 0157 (109) occasionally have been isolated from calves, but they appear to be much less common than those in the table. The serogrouping information in other less detailed reports (4, 33, 34, 99, 122) is generally consistent with that shown here.




<sup>b</sup>NE = Not examined.

<sup>c</sup>(A) = Presence of an A-type K antigen that could not be typed with existing K antisera.

<sup>d</sup>S.D. Acres, unpublished data. Serotyping on these strains was kindly performed by I. and F. Ørskov, International Escherichia and Klebsiella Center, Copenhagen, Denmark.

whether they are expressed by ETEC under the environmental conditions that exist in the small intestine or whether they are important attachment factors in vivo (60). Therefore, although K99 is the most common attachment factor on bovine ETEC, it appears that there may be a relatively small group of other fimbriae that are also attachment factors for calf intestine. This is not surprising, as a similar situation occurs in ETEC isolated from pigs where K99, F41, and at least two other fimbriae, known as K88 and 987P, are important (6, 92).

The nomenclature for fimbriae (pili) is confused. Recently, it was suggested that the term fimbriae should replace the term pili and that various fimbrial (F) antigens be designated systematically by number as with the classical O:K:H antigens (23, 61, 103). Under this new scheme K99 would be designated F5 (103). Hence, the currently used serotype designation 09:K30:K99:H- would be replaced with 09:K30:H-:F5. The other fimbriae on calf ETEC have not yet been assigned an F number within the proposed scheme.

Figure 3. Electron micrograph of a section of ileum from a calf infected with enterotoxigenic *Escherichia coli* strain B44. The tissue was immersed in K99 monoclonal antibody for 1 h before staining with ruthenium red. The K99 fimbriae are thickened by the reaction with antibody (  ) and appear as electron-dense projections extending from the bacterial cell surface, through the capsule, to reactive sites on microvilli of the intestinal epithelial cells (  ). The capsular exopolysaccharide has collapsed during processing onto the fimbriae to form amorphous accretions on the bacterial cell surface and the fimbriae (  ). (Bar = .1 μ).





### Capsular (K) Antigens

Capsular (or K antigens from the German word Kapsel) are fibrous projections composed of acidic polysaccharides that surround or encapsulate the somatic antigen on some but not all ETEC. Under the electron microscope they appear as a dense mat of fibrous projections and are similar to the glycocalyx on other bacteria (Figures 4 and 5). Capsules originally were divided into L, A, or B subtypes based on their behavior in serological tests after heating at various temperatures; however, the original criteria for separating K antigens into these three subtypes are no longer valid (104). However, the designation of type (A) has been retained for K antigens that designate discrete capsules that occur in combination with somatic (O) antigens 8, 9, 20, and 101 (104). There are approximately 70 K groups in total (104); however, only 11 have been identified commonly on calf ETEC (Table 1), and most of these are of the (A) type. Several other unidentified groups, designated as (A) in Table 1, probably occur, but no attempt was made to type them, or they were unable to be typed with antisera against any of the existing groups. Hence, some may represent new antigens not yet included in the international serotyping scheme. Therefore, a dozen or more capsular groups may occur on calf ETEC. The exact role of capsular antigens in the pathogenesis of diarrheal disease is not clear; however, they probably aid in colonization by protecting the bacteria against immune mechanisms in the intestine and by reinforcing fimbrial attachment to the intestinal mucosa (16, 17, 49, 50, 60, 138).

### Somatic (O) Antigens

Serologically, the somatic antigen represents the O-specific polysaccharide of the cell wall lipopolysaccharide on all smooth colony-type *E. coli* (90). Approximately 160 O groups have been identified, but only groups 8, 9, 20, 26,

101, and 141 are common on calf ETEC (Table 1). The role of somatic antigens in the pathogenesis of diarrhea is not clear; however, it is assumed that the strains that possess these particular O groups offer advantages to the plasmids that carry the genetic material encoding for enterotoxin and fimbrial production (35).

Table 1 shows that there is a close relationship between the occurrence of STa, K99, and to less extent F41, and the OK serogroups on calf ETEC. Production of STa and K99, and probably other fimbriae, is controlled by genes located on transmissible plasmids, which are extra chromosomal pieces of DNA in the cytoplasm of the bacterial cell (Figure 1) (133, 138, 140, 149). This indicates that the virulence plasmids are accepted and retained more readily by *E. coli* with the specific characteristics associated with the O and K antigens in Table 1, and it has been suggested that these serotypes serve as "reservoirs" for the plasmids (35). Bacterial geneticists have taken advantage of the transmissible nature of the plasmids that control production of STa and K99 to help to define the role of each factor in the pathogenesis of diarrhea. By creating bacterial mutants that possess various combinations of these two factors, they have shown that both STa and K99 are necessary to cause disease. Strains of *E. coli* that produce only one or the other do not cause diarrhea (138, 140). It is likely that some or all of the other fimbriae mentioned also act as attachment factors either singly or in combination with K99 (19, 92, 109, 110). If so, it is likely that K99-negative ETEC eventually will be confirmed as causes of diarrhea in calves.

### PATHOGENESIS OF ENTERIC COLIBACILLOSIS

The main features in the pathogenesis of ETEC disease are: 1) infection with ETEC, 2) attachment of ETEC to epithelial cells resulting in colonization of the small intestine,

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Figure 4. Electron micrograph of a section of ileum from a calf infected with enterotoxigenic *Escherichia coli* strain B44. The tissue was immersed in anti-K30 antibody to stabilize the capsule prior to staining with ruthenium red. The capsule appears as a well-consolidated electron-dense structure (  $\blacktriangleright$  ) enclosing microcolonies of bacteria attached to microvilli of epithelial cells (  $\blacklozenge$  ) and providing reinforcement and structural rigidity to the fimbriae, which are not visible in this preparations. (Bar = .1  $\mu$ ).



and 3) production and action of STa. This chain of events leads to an acute profuse watery diarrhea culminating in dehydration, metabolic acidosis, and death in severe cases.

#### Infection with Enterotoxigenic *Escherichia coli*

Calves become infected with *E. coli* during or shortly after birth, often by fecal-oral transmission (132). Under normal circumstances, nonpathogenic types of *E. coli* are one of the first bacterial species to infect the gastrointestinal tract, and they are present throughout the gut by the end of the 1st day of life (132). Rapid establishment of *E. coli* is favored by several characteristics of newborn calves including a relatively high abomasal pH, sluggish intestinal motility, and absence of competing microflora. The majority of these nonpathogenic *E. coli* are suspended in intestinal contents and are constantly propelled caudally by peristalsis and flow of ingesta; hence, their numbers rarely exceed  $10^7$ /g of intestinal contents (132). However, when ETEC are ingested, they multiply and colonize the small intestine in much larger numbers.

#### Colonization

Colonization of the posterior half of the small intestine is the central event in pathogenesis of enteric colibacillosis. In the ileum there may be as many as  $10^9$  or  $10^{10}$  ETEC/g of intestinal contents (86, 138). Although it is a complex process not completely understood, attachment of ETEC to the intestinal mucosa is the main mechanism and the one that allows bacteria to overcome the natural peristaltic cleansing action of the intestine. Hence, colonization involves a marked increase of the number of ETEC as well as the proportion that are attached to the intestinal mucosa (49, 50, 138, 140). In normal calves, the majority of the *E. coli* are in the luminal contents, and only 10 to 20% are attached to the mucosa. In contrast, in calves with enteric colibacillosis the situation is reversed, and 80 to 90% of the ETEC are attached (49, 50). The dynamics of this process have been studied in colostrum-deprived calves where it appears that colonization begins at the ileal-cecal junction within 3 h of infection and progresses anteriorly to involve up to 60% of the small intestine by 16 h after infection (108).

The precise mechanism of attachment at the molecular level has not been established; however, bacterial fimbrial and capsular antigens both appear to be involved (16, 49, 50, 138). Available information suggests the following sequence of events. The surface of bacterial and intestinal epithelial cells both are charged negatively and, hence, there is a natural tendency for them to repel each other. Fimbriae, such as K99, protrude from the bacterial cell surface and initiate attachment by extending across the zone of repulsion to reach specific receptor sites on the villous epithelial cells (Figure 3). Receptors are probably sugar residues located in the cell membrane of the epithelial cells (36), and those which bind to K99 and F41 fimbriae appear to contain sialic acid (68). Following primary attachment the bacteria multiply and form microcolonies that cover the surface of the villi and that are sometimes several layers thick (Figure 4) (8, 16). The ETEC do not appear to attach to crypt epithelial cells.

Although capsules are not a prerequisite for colonization, they are on most ETEC, and some K serogroups probably aid in pathogenesis both by protecting the bacteria against host immune responses and by reinforcing primary fimbrial-mediated attachment (16, 17). This is suggested by electron microscopic studies of intestinal tissue taken from calves infected with encapsulated strains. When intestinal tissue is treated with anticapsular antibody and stabilized with ruthenium red dye prior to fixation, the role of the capsule in colonization can be visualized. These studies illustrate that large amounts of capsular material are produced in vivo and appear to encase and cement microcolonies of ETEC onto the mucosal surface, thereby protecting them from fluid movement and phagocytosis (Figures 4 and 5) (16, 17, 50). In such cases, the outer boundary of the capsular material is in intimate contact with the brush border of the epithelial cells (Figure 5). It has been suggested that capsules may confer other advantages on the bacteria by directing toxins to the target epithelial cells and by channeling nutrients available at the cell surface to the bacteria (22).

The degree of intestinal colonization varies among strains of ETEC (Figure 6) and probably is affected by serotype (17, 49, 50, 66) as well as physiological and nutritional factors in the small intestine (26, 57, 64). Maximum col-

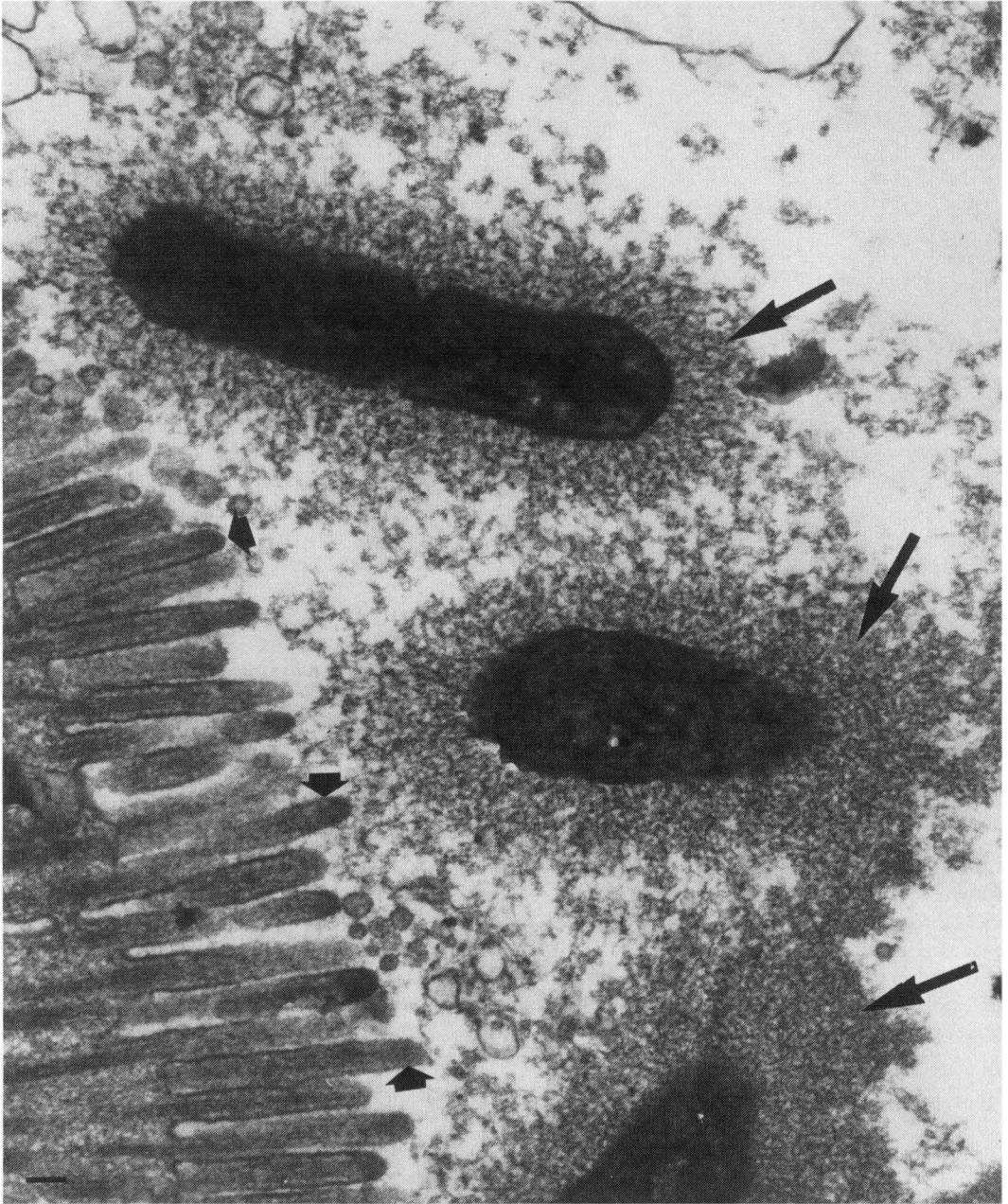


Figure 5. Electron micrograph of a section of ileum from a calf infected with enterotoxigenic *Escherichia coli* strain B44. The tissue was immersed in anti-K30 antibody to stabilize the capsule prior to staining with ruthenium red. The fibrous structure of the capsular glycoalyx (  $\blackrightarrow$  ) and the intimate contact between it and the epithelial cell surface (  $\blacklozenge$  ) can be seen at this magnification. The capsule probably reinforces the primary fimbrial-mediated attachment and protects the bacteria against mechanical and immunological protective mechanisms which are active at the mucosal surface. (Bar = .1  $\mu$ ).

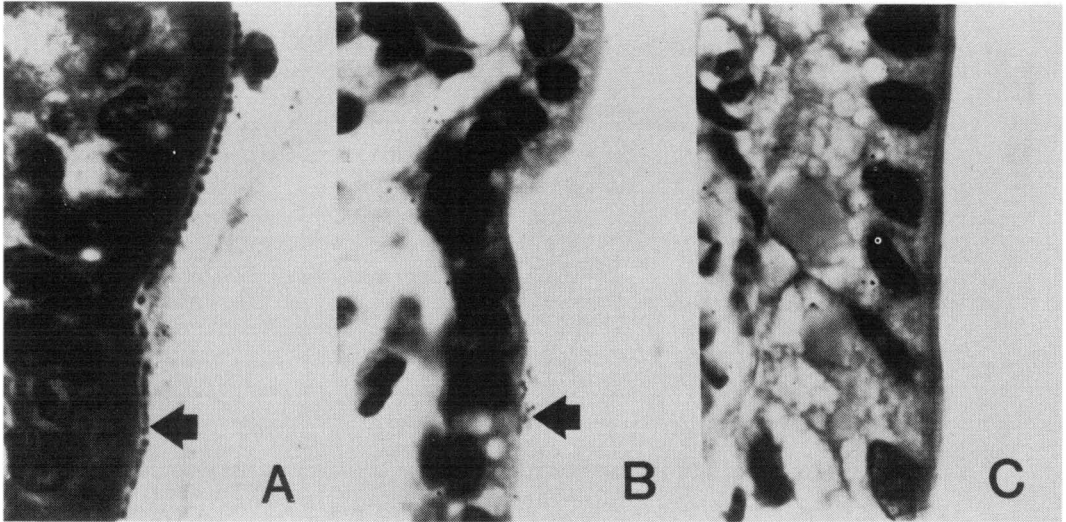


Figure 6. Light micrograph of sections of tissue taken from the ileum of newborn calves for histological examination: A) Colostrum-fed calf infected with enterotoxigenic *Escherichia coli* strain B44 (09:K30:K99:F41:H-). A continuous layer of bacteria cover the surface of villous epithelial cells (8). B) Colostrum - deprived calf infected with ETEC strain 210 (09:K30:F210:H-). Attachment is sparse and focal (9). C) Uninfected control calf. The surface of the epithelial cells is not colonized. (Reprinted from Can. J. Comp. Med. 47:143 with permission.)

onization seems to occur with strains that are both fimbriated and encapsulated (49, 138). In addition, some strains possess only one type of fimbriae, whereas others possess more than one type (19, 88, 104, 109), which may intensify colonization and increase virulence.

In summary, the initial step in attachment appears to be a function of fimbriae which attach to specific receptors on the mucosa. In some cases, secondary reinforcement is provided by capsular glycocalyx which cements microcolonies of bacteria onto the epithelial cell and places the outer boundaries of the two cell types in intimate contact.

#### Production and Action of Enterotoxin

When large numbers of ETEC colonize the small intestine, sufficient STa is produced to cause diarrhea. Presumably, enterotoxin is released by the bacteria and diffuses toward the brush border where it binds to receptor sites on the membrane of the intestinal epithelial cells (Figure 1). The enterotoxin alters normal movement of ions and water across the intestinal mucosa by exerting a hormone-like

effect on the enterocytes (80). Fluid and electrolytes constantly move across the intestinal mucosa in two directions: from intestinal lumen to blood (absorption), and from blood to intestinal lumen (secretion). Secretory and absorptive fluxes occur simultaneously, and the net movement of fluid is the difference between the two processes. The villus-crypt unit is the functional apparatus through which these fluxes occur (80). Immature enterocytes in the crypt are the main secretory cells, whereas the more mature villous enterocytes are responsible for digestion and absorption. In the normal calf, absorptive fluxes exceed secretory fluxes creating a state of net absorption (80). The STa appears to act by stimulating secretion as well as by reducing absorption (37), and this dual effect reverses the normal pattern causing a net secretion of fluid into the small intestine and resulting in hypersecretory diarrhea (Figure 7).

The total volume of each unidirectional flux in normal calves 4 to 6 wk of age is between 80 and 144 liters/day (14). Calves infected with ETEC may lose between 1.0 and 2.7 liters of diarrheal fluid in 24 h (38, 72), which represents

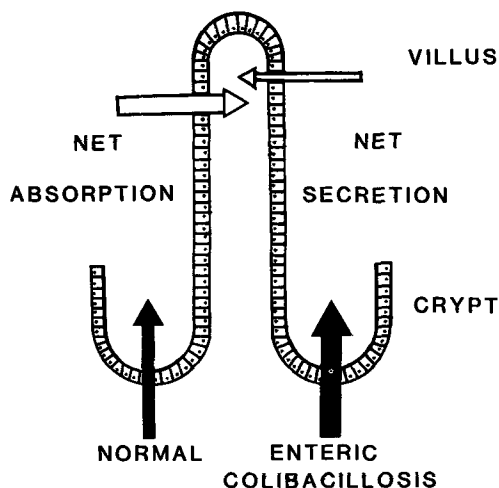


Figure 7. Schematic drawing of crypt-villous unit in the small intestine. Fluid and electrolytes are secreted by the immature crypt epithelial cells and absorbed by the mature villous epithelial cells. Left: in the normal calf, the volume of fluid absorbed from intestinal lumen into the circulation (⇨) exceeds the volume secreted from blood to lumen (⇩). Hence, there is net absorption of fluid. Right: in a calf colonized by enterotoxigenic *Escherichia coli*, there is hypersecretion of fluid and electrolytes by crypt cells (⇩) and decreased absorption by villous cells (⇨) in response to enterotoxin. Hence, there is net secretion of fluid into the intestinal lumen.

only 1 to 2% of the normal unidirectional flux volume. This illustrates that only a small percentage change of the normal capacity of the small intestine is required to cause severe diarrhea (15).

The detailed biochemical and physiological actions of STa have not been determined clearly, but a working hypothesis can be developed from existing data (Figure 8). Primary events in hypersecretion appear to involve activation of guanylate cyclase which increases guanosine 5'-monophosphate (GMP) (37, 43, 101, 118), and an increase of intracellular calcium ions probably as a result of opening of calcium gates in the cell membrane (145). This dual event could imply involvement of phosphatidylinositol hydrolysis as parallel activation of guanylate cyclase and phosphatidylinositol hydrolysis-dependent calcium entry into cells occurs in a variety of cholinergic-muscarinic receptor systems (76). As a result,

there may be activation of the calcium-binding protein calmodulin followed by release of arachidonic acid, which acts as a substrate for prostaglandin synthesis (145), and production of diacylglycerol, which stimulates protein phosphorylation by activating c kinase. Any or all of these events could be capable of stimulating hypersecretion. Increased cyclic GMP also is believed to block absorption of chloride ions and water by mucosal cells (37). Regardless of the specific pathways involved, the end result is to reverse the usual physiologic state of the small intestine from one of net absorption to one of net secretion of fluid. Excess fluid accumulates in the lumen and eventually is lost as diarrheal feces.

**TRANSMISSION OF ENTEROTOXIGENIC *Escherichia coli***

Infected animals are the main reservoir for ETEC, and their feces are the major source of environmental contamination with the bacteria (150). Passage of ETEC through animals causes a "multiplier effect", as each infected animal excretes many more bacteria than it originally ingested (20). Diarrheic calves are the most extreme multipliers, because they often pass 1,000 ml or more of diarrheal feces containing 10<sup>10</sup> ETEC/ml within 12 h, and recovered calves can continue to shed the bacteria for several months (150). Normal calves that are infected subclinically also can excrete significant numbers of ETEC. For example, colostrum-deprived calves that were infected experimentally with as few as 70 ETEC of serogroup

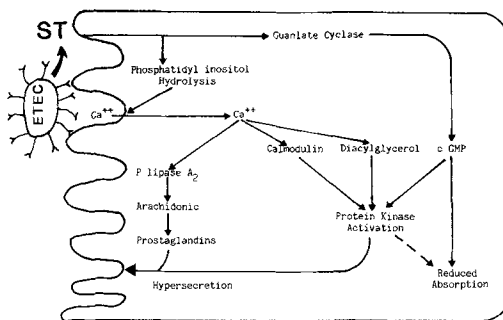


Figure 8. Hypothetical biochemical pathways by which *Escherichia coli* heat stable enterotoxin stimulates hypersecretion by crypt epithelial cells and reduced absorption by villous epithelial cells.

09:K35:K99 remained clinically normal but shed up to  $10^6$  ETEC/g of feces for several days (150). Adult animals also can serve as a reservoir for infection, and recent studies in one dairy herd showed that 15 of 152 (10%) cows were shedding  $10^2$  to  $10^4$  ETEC/g of feces when sampled within 1 wk of parturition (150). Thus, tremendous multiplication of ETEC occurs in animals clinically as well as subclinically infected, and the bacteria can persist in a herd by circulating through animals of all ages. Carrier animals introduced to an uninfected herd are thought to be one of the main causes of natural outbreaks. Duration and amount of shedding probably depend on the degree and duration of confinement, resulting population density, herd immunity, environmental conditions, and perhaps serotype of ETEC.

The ETEC are relatively resistant to environmental factors and can survive for long periods under the right conditions. One recent study showed that ETEC of serogroup 09:K35:K99 survived as long as 6.5 mo in calf crates contaminated with diarrheic feces (150). The bacteria may persist even longer under ideal conditions of temperature and relative humidity. The survivability of different types of ETEC in the environment has not been studied; however, heavily encapsulated strains may resist dehydration and exposure to radiation more than nonencapsulated strains.

#### INCIDENCE OF ENTEROTOXIGENIC

##### *Escherichia coli*

Results of field surveys on the incidence of ETEC, selected because they involved predominantly dairy rather than beef calves, are summarized in Table 2. Most calves in these studies were diarrheic and under 3 wk of age when examined. Collectively these results provide an estimate of the incidence of ETEC alone or in mixed infections as compared with other enteropathogens such as rotavirus, coronavirus, and cryptosporidia. The incidence of ETEC varies widely within herds and within individual calves and probably is affected by geographic, management, seasonal, and diagnostic variables. Nonetheless, the composite results indicate that ETEC are not the most common enteropathogen and rarely are isolated from more than 25 to 30% of diarrheic calves during the first 3 wk of life. Other entero-

pathogens, either separately or in combination with each other, occur in a much higher percentage of calves. However, ETEC appear to be one of the most common causes of diarrhea during the first 3 or 4 days of life, and they have been found in up to 50 to 60% of calves in this age group (4, 87, 89). Hence, ETEC are more prevalent in young calves, and the average age at onset of diarrhea caused by ETEC is under 2 days, whereas it is greater than 6 days when other enteropathogens are involved (82, 87). Even though ETEC are not as common as other enteropathogens, particularly rotavirus, they cause a more severe diarrhea (77). In a recent study, 29% of the dairy calves infected with K99<sup>+</sup> ETEC, as compared with only 5% of calves infected with rota or corona viruses, or both died or were severely ill (77). Hence, although herd outbreaks of ETEC diarrhea tend to be infrequent, they can be severe and cause substantial economic loss to dairy producers.

#### DIAGNOSIS OF ENTERIC COLIBACILLOSIS

Diagnosis of diarrhea caused by ETEC should be based on qualitative as well as quantitative characterization of the *E. coli* population in the small intestine (66, 82, 85, 86, 87). The common practice of simply culturing feces or intestinal contents for *E. coli* is meaningless unless bacterial isolates also are examined for specific virulence determinants characteristic of ETEC. To detect K99 antigen on bacteria grown in culture, special media that promote expression of the fimbriae should be used (40, 47), because some nutrients, such as alanine (26) in commonly used bacterial media, are inhibitory. The isolation rate of K99<sup>+</sup> strains increases significantly when these media are used [see Table 2 and (64)]. Assays also have been developed to detect isolates that produce STa (1, 33, 53, 85, 128). In spite of the usefulness of these assays, isolation of K99<sup>+</sup>, STa<sup>+</sup>, or both bacteria from feces or small intestinal contents of diarrheic calves provides only presumptive evidence of their role in causing diarrhea and can be misleading because small numbers of ETEC occasionally can be isolated from healthy calves (82, 85). Therefore, their importance should be confirmed by demonstration that they have colonized the small intestine in large numbers. Isolation of ETEC in



TABLE 2. Incidence of enterotoxigenic *Escherichia coli* (ETEC) and other enteropathogens in diarrheic dairy calves.

Enteropathogen	Study	Herds	Percent	Calves	Percent	Country	Reference
ETEC <sup>a</sup>	1	NS <sup>b</sup>	NS	6/55 <sup>c</sup>	11	Canada	86
	2	NS	NS	9/51	18	Canada	87
	3	1/12 <sup>c</sup>	8	2/32	6	USA	82
	4	NS	NS	24/194	12	Canada	66
	5	0/6	0	0/86	0	Scotland	141
	6	17/54	31	17/54	31	USA	85
	7	NS	NS	55/200	28	Canada	129
	8 <sup>d</sup>	NS	NS	12/237	6	Israel	64
		NS	NS	209/503	41	Israel	64
9	18/20	90	49/226	22	USA	52	
ETEC Mixed with other <sup>e</sup>	1	NS	NS	4/55	7		86
	2	NS	NS	6/51	12		87
	3	4/12	33	7/32	22		82
	4	NS	NS	12/194	6		66
	5	0/6	0	0/86	0		141
Other <sup>e</sup>	1	NS	NS	25/55	45		86
	2	NS	NS	33/51	65		87
	3	7/12	58	15/32	47		82
	4	NS	NS	118/194	61		66
	5	6/6	100	64/90	71		141

<sup>a</sup>In studies 1 to 5, diarrheic calves were examined for a variety of enteropathogens and were diagnosed as infected with ETEC only, ETEC in combination with other enteropathogens, or other enteropathogens. In studies 6 to 9, calves were examined only for ETEC; hence, mixed infections would not have been detected. Therefore, in the four latter studies the proportion of ETEC-infected herds and calves would be equivalent to those classified as infected with ETEC, plus ETEC mixed with other enteropathogens in the top five studies. In (82), every calf was not examined for all enteropathogens.

<sup>b</sup>NS = Not studied or not reported.

<sup>c</sup>Number positive/number tested.

<sup>d</sup>This survey was conducted for 3 yr. The isolation rate of ETEC increased significantly during the 2nd and 3rd yr when selective media for K99 antigen was used.

<sup>e</sup>Other enteropathogens include rotavirus, coronavirus, cryptosporidia, and in some studies *Salmonellae*, bovine virus diarrhoea, and infectious bovine rhinotracheitis.

pure culture or as the dominant aerobic flora recovered from feces or intestinal contents on unselective bacterial media is one indication that they are numerous. A variety of other techniques, including counting the number of bacteria per gram of intestinal contents or subjectively estimating the number of bacteria in mucosal smears also have been used (64, 66, 86, 87). The most direct evidence for colonization can be provided by microscopic examination of intestinal tissue prepared for routine histopathological examination (Figure 6) or stained with fluorescein-labeled K99 antiserum. Histopathologic changes character-

istic of ETEC infection include a layer of Gram-negative bacteria adhering to the mucosa (Figure 6), stunted villi covered by cuboidal epithelium in the ileum and jejunum, and accumulations of neutrophils above the dome area of Payer's patches (8, 9). However, these lesions are detected most easily when calves are submitted alive and when tissues are taken within a few minutes of sacrificing them because postmortem changes of the mucosa occur rapidly (107). Fluorescent antibody staining of intestinal tissue has several advantages: it identifies K99 antigen, layers of bacteria attached to the villus can be visualized,

and difficulties with identifying the antigen in strains grown in culture are avoided (82, 85). In herds where mixed infections occur, it is often necessary to examine several calves before all the various enteropathogens are detected. Therefore, in problem herds the diagnosis of diarrheal disease should be based on examination of a number of calves and should include techniques that will identify a variety of enteropathogens (82, 85).

#### NATURAL RESISTANCE TO ENTERIC COLIBACILLOSIS

Diarrhea caused by ETEC occurs in calves mainly during the first few days of life, rarely in older calves, and never in adults. Epidemiological studies of both beef and dairy calves indicate that more than 80% of clinical cases caused by K99<sup>+</sup> ETEC occur in calves younger than 4 days of age (4, 29, 74, 77, 128).

These epidemiological observations are supported by results of experimental studies in which calves were challenge-inoculated orally with ETEC at various ages. Calves inoculated on the 1st day of life almost always developed diarrhea, and colonization of the posterior half of the small intestine was evident at postmortem examination. In contrast, calves inoculated after the 1st day of life showed increasing resistance to diarrhea which was complete by 48 to 96 h of age (135). The mechanism of this age-related resistance is not known. It is not mediated by passive antibody because colostrum-deprived calves are also refractory to challenge by the 2nd or 3rd day of life (135). The decrease of abomasal pH soon after birth does not appear to be involved, because ETEC can survive pH as low as 2.5 (131) and can be recovered from the small intestinal contents of nonsusceptible calves. Similarly, loss of responsiveness to STa is an unlikely mechanism because STa-induced hypersecretion occurs in calves as old as 2 wk of age (13). These observations suggest that the mechanism involves development of resistance to colonization of the small intestine, and this hypothesis is supported by *in vitro* studies that show that intestinal cells develop resistance to K99-mediated attachment as calves become older (119). Hence, it appears that the K99-sensitive villous epithelial cells present at birth either lose their K99 receptors or are replaced by a

new population of cells that lack receptors. Therefore, calves are susceptible to colonization by K99<sup>+</sup> ETEC for only a brief period immediately after birth.

In those cases where ETEC are isolated from older calves, they are usually in combination with one or more other enteropathogens such as rotavirus (82, 85, 87). In some mixed infections involving rotavirus and ETEC, large numbers of the bacteria attach to virus-infected mucosa (87). This suggests that the virus has altered the small intestinal epithelium, thereby prolonging the normal period of susceptibility to colonization, or that the ETEC are attaching by some mechanism other than K99, perhaps by other fimbriae or capsules. In other cases, ETEC are numerous in the lumen, but there is little attachment (142, 149, 148), and the significance of the bacteria is not clear. Presence of ETEC probably increases the severity of rotavirus diarrhea; however, the extent of the interaction appears to vary with serotype of ETEC, strain of rotavirus, and age and immune status of calves (120, 142, 147, 148).

The possibility of breeding animals that are resistant to colonization by ETEC has been explored in pigs. This was made possible after observations on susceptibility of individual litters to natural and experimental infections, selective breeding experiments, and phenotyping of parents and their offspring revealed that there are two phenotypes of pigs for presence of intestinal receptors for K88<sup>+</sup> ETEC (45, 123, 124, 125, 126). The two phenotypes are products of two alleles at a single locus that are inherited in a simple Mendelian manner. Adherence (S) is dominant over nonadherence(s). Homozygous dominant (SS) and heterozygotic (Ss) pigs have K88 receptors on their enterocytes and are susceptible to colonization by K88<sup>+</sup> ETEC. In contrast, pigs of the homozygous recessive phenotype (ss) lack intestinal receptors and are resistant to colonization and diarrhea. In herds infected with K88<sup>+</sup> ETEC, matings between resistant dams (ss) and susceptible boars (SS or Ss) produce the most susceptible offspring because the resistant dams do not produce protective antibodies (123, 125). A similar genetic basis for K99-receptor sites has not been reported in calves.

### IMMUNITY TO ENTERIC COLIBACILLOSIS

Mammary secretions contain a variety of nonspecific and specific factors that potentially can prevent bacterial infections (Table 3). The activity of some of these factors against ETEC has been demonstrated whereas effects of others have not.

#### Nonspecific Immunity

Colostrum from unimmunized cows provides some protection against ETEC; however, it is not comprehensive and often fails to protect calves born and raised under modern husbandry systems. Results of recent experimental studies suggest reasons. Using a strain of ETEC of serogroup 0101:K(A), Logan et al. showed that calves fed nonimmune colostrum 2 h before experimental challenge were not colonized and did not develop diarrhea, whereas those fed 2 to 6 h after challenge were colonized, became diarrheic, and developed intestinal lesions similar to those in colostrum-deprived calves (71). Thus, it appears that time of colostrum feeding relative to time of infection is an important factor and that colostrum must be consumed before infection occurs to establish a protective immunological barrier in the small intestine. In addition, other experiments have shown that protection provided by early consumption of nonimmune colostrum can be overwhelmed by feeding relatively large doses of ETEC (2, 3, 8, 97, 98). For example, even calves that nursed initially within 1 to 2 h after birth and several more times before subsequent challenge at 12 to 14 h of age with  $10^{11}$  ETEC, remained susceptible to fatal diarrhea (2). Therefore, there is a balance among the time at which colostrum is ingested, volume ingested, and number of ETEC to which calves are exposed. Husbandry practices that cause delayed or inadequate colostrum consumption or exposure to a highly contaminated environment nullify natural protection and foster outbreaks of ETEC diarrhea.

*Mechanisms of Nonspecific Immunity.* Mechanisms of protection provided by colostrum from nonimmune cows is not well characterized. Logan et al. showed that feeding colostrum prior to challenge prevented diarrhea, but that immunoglobulins IgG, IgM, and IgA fractions prepared from whey and fed separately only reduced the severity of diarrhea but did not

prevent it (72). These results suggest that at least part of the protection was due to immunoglobulins but that other factors also may have contributed. Colostrum in these experiments contained antibody against the somatic antigen of the challenge strain but apparently was not examined for antibodies to other antigens. Because evidence for the protective capacity of somatic antibodies is inconclusive (*vide infra*), the mechanism of protection was not identified clearly. Others also have found that a variable proportion of calves were resistant to experimental infection even though no antibodies to the challenge strain were detected in colostrum of their dams (2, 94). There are several explanations for these observations including: 1) lack of sensitivity of the antibody assays, 2) presence of antibodies to antigens other than those measured, such as unidentified fimbriae, capsules, enterotoxin, other bacterial antigens (*vide infra*), or endotoxin, 3) nonantibody protective factors, or 4) genetically determined absence of bacterial receptors for K99 antigen mucosa of some calves.

The nonantibody bacterial inhibitors in mammary secretions include the iron-binding proteins lactoferrin and transferrin (11, 67, 114, 115) the lactoperoxidase-thiocyanate-hydrogen peroxide (LP) system (114, 116, 117), and factors that are structurally analogous to receptor sites on intestinal epithelial cells for fimbriae or enterotoxin (Table 3).

Bacteriostatic effects of lactoferrin and transferrin are due to the ability of these

TABLE 3. Nonspecific and specific immune mechanisms that may contribute to protection against enterotoxigenic *Escherichia coli* infections in calves.

- 
- |  |
|--|
| A. Nonspecific mechanisms  |
| 1. Lactoferrin and transferrin   |
| 2. Lactoperoxidase-thiocyanate-hydrogen peroxide system                    |
| 3. Analogues for fimbrial or enterotoxin receptor sites                    |
| B. Specific mechanisms   |
| 4. Antiadhesive antibody   |
| 5. Antitoxic antibody  |
| 6. Bacteriostatic or bactericidal antibody                                 |
| a) antibody + complement   |
| b) antibody + lactoferrin  |
| 7. Antibody-induced elimination of virulence determinants (plasmid curing) |
-

proteins to bind iron, which is an essential nutrient for bacterial growth (12). Iron binding is promoted by bicarbonate and inhibited by citrate and enterobactin, an iron chelating compound produced by *E. coli* (12, 115). Colostrum contains relatively high citrate (3.6 mg/ml), which inhibits the action of lactoferrin. However, when bicarbonate is added or citrate is removed, colostrum contains sufficient lactoferrin (1 to 4 mg/ml) to inhibit growth of ETEC in vitro (114). Reiter suggested that citrate in colostrum is absorbed in the small intestine and that sufficient bicarbonate is added from the saliva and intestinal secretions of calves to allow lactoferrin-mediated bacteriostasis to occur in vivo (Figure 9B) (114). However, the importance of this mechanism against ETEC in new born calves raised under natural conditions has not been determined.

The LP system also may provide protection against bovine ETEC provided that all three components of the system are present. Lactoperoxidase and thiocyanate are provided to the newborn calf in colostrum and milk (116, 117). Hydrogen peroxide is produced by lactobacilli (117) that are not in the abomasum until the end of the 1st day of life (132); therefore, this system may not be active immediately after birth. This system is most active in the abomasum and upper small intestine, and both bacterial killing (Figure 9B) and inhibition of ETEC attachment have been demonstrated in vitro (114, 117).

Other nonimmune factors in mammary secretions that could inhibit ETEC infections are structural analogues for the fimbrial and enterotoxin receptor sites on epithelial cells. These receptor analogues effectively could block pathogenesis by binding to fimbriae or enterotoxin before they reach the brush border (Figure 9C). For example, the membrane of sow milk fat globules contains a mucoprotein receptor that closely resembles the receptor on piglet enterocytes for K88 fimbriae (111, 114). This swine-associated mucoprotein (SWAMP) blocks attachment of K88<sup>+</sup> ETEC to piglet enterocytes in vitro, possibly by steric hindrance, and may have a similar function in vivo. Fat globules of cow's milk also contains a bovine-associated mucoprotein (BAMP) (112) that could act in a similar manner to block attachment of K99<sup>+</sup> or other fimbriated ETEC in calves although this possibility has yet to be

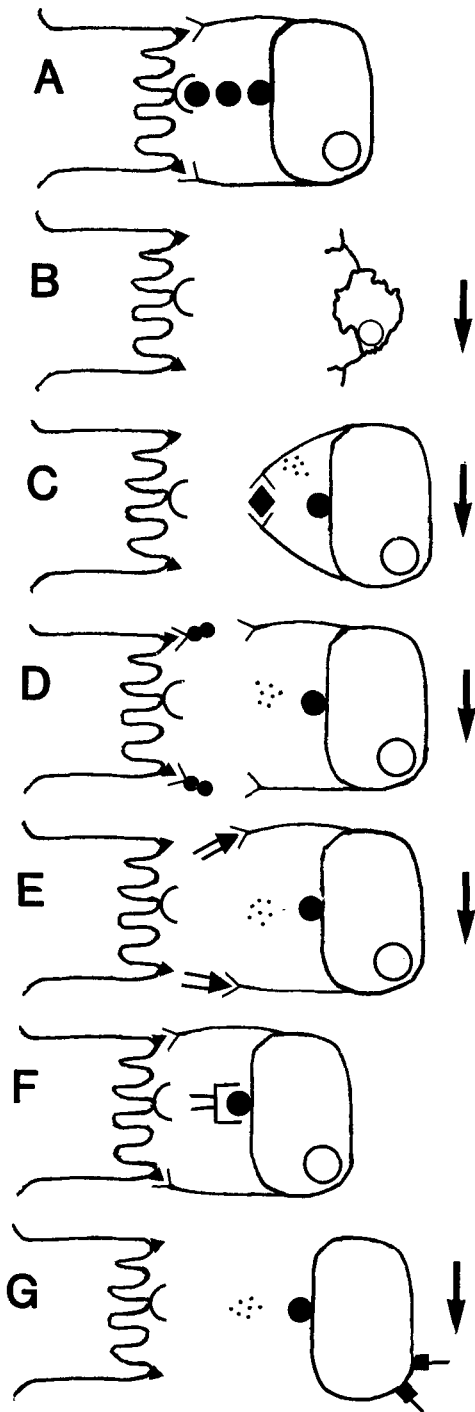
investigated. Similarly, analogue receptors for *E. coli* heat labile enterotoxin recently have been described in human milk which raises the possibility that similar factors for STa could be in bovine milk (54, 106).

In addition to receptor analogues mentioned, another possible method of blocking bacterial adherence is by flooding the small intestine with an excess of fimbriae or fimbrial analogues (Figure 9D). These would bind to the receptor sites thereby making them unavailable for attachment by fimbriae on ETEC. This method was used experimentally by Davidson and Hirsch, who showed that feeding a K88<sup>+</sup>, nonenterotoxigenic strain of *E. coli* reduced the severity of diarrhea caused by a K88<sup>+</sup> ETEC strain (25). This procedure has not been used in calves or piglets raised under natural conditions.

For a variety of reasons, many calves born and raised under modern husbandry conditions do not ingest sufficient colostrum soon enough after birth to protect them against the number of ETEC in their environment. Hence, the nonspecific protective mechanisms often are overwhelmed, and calf losses continue. Therefore, considerable effort has been directed toward developing immunological methods of preventing and controlling ETEC. Discoveries in two general areas have encouraged these developments. First, the observation that calves are susceptible to colonization by ETEC for only a brief period immediately after birth suggested that it might be possible to prevent the disease by stimulating formation of protective antibodies in colostrum that would be transferred to the small intestine of calves during the period of susceptibility. Second, insight into the structure of ETEC and the function of fimbriae in the pathogenesis of diarrhea helped to identify candidate antigens that would induce specific protective antibodies.

#### Specific (Antibody-Mediated) Immunity

Neonatal calves depend on antibody derived from mammary secretions for prevention against systemic as well as localized *E. coli* infections. Colostrum antibodies that are absorbed from the lumen of the small intestine into the systemic circulation during the first 24 h of life prevent generalized colisepticemia but provide little or no protection against ETEC. Conversely,



passive antibodies that remain in the lumen of the small intestine or are adsorbed onto the surface of villous epithelial cells prevent ETEC infection but not colisepticemia (72, 139). Hence, it is not uncommon for calves to be protected against colisepticemia but still to succumb to severe enteric disease.

#### Immunizing Properties of Enterotoxigenic *Escherichia coli* Antigens

Immunizing properties of several types of ETEC antigens have been examined (79). These include somatic, capsular, and fimbrial antigens (Table 1) as well as STa. Most of the evidence regarding the protective capacity of these antigens has been obtained from vaccination-challenge experiments in which pregnant cows were vaccinated either subcutaneously or

Figure 9. Schematic representation of some of the nonspecific and specific mechanisms that could protect against enterotoxigenic *Escherichia coli* infections: A) Bacteria attach when specific binding sites on fimbriae (>) contact receptor sites on the intestinal epithelial cell (>•). This leads to colonization of the small intestine and release of enterotoxin (•), which also binds to receptor sites (◁) on the epithelial cells. Diarrhea results. B) Enterotoxigenic *E. coli* can be killed or inhibited by a variety of factors in lacteal secretions including lactoferrin, the lactoperoxidase-thiocyanate-hydrogen peroxide system, and perhaps antibody against a variety of bacterial antigens. C) Bacterial attachment (or enterotoxin binding) can be blocked by factors in lacteal secretions which are structurally and functionally analogous to fimbrial receptor sites (♦) on epithelial cells surface, for example, milk fat globule mucoproteins. The bacteria do not attach to the intestinal epithelium and are swept away by the movement of ingesta down the lumen (⇨). D) Bacterial attachment can be prevented by flooding the intestine with fimbriae or fimbrial analogues (>••), which block access to the receptor sites on the intestinal epithelial cells. One method of achieving this is to feed strains of nonenterotoxigenic *E. coli* which carry attachment fimbriae (25). E) Bacterial attachment to epithelial cells can be blocked by anti-fimbrial antibody (⇨). F) Enterotoxin can be neutralized or blocked by antibody (⇨). G) Antibody (■) against unknown bacterial antigens can cause loss of plasmids (○) that carry the genes responsible for production of fimbriae. As a result of this "plasmid curing" the attachment factors are also lost and colonization does not occur. Enterotoxin (•) may be produced but it dissipates (∴) before reaching receptor sites in sufficient quantity to stimulate secretion.



intramuscularly during the final trimester of gestation to stimulate formation of serum antibodies that are concentrated in colostrum before parturition. After birth, calves were allowed to ingest colostrum and then were challenged with virulent ETEC at 10- to 18-h-old. The degree of protection was determined by evaluating the relationship between colostral antibody titer and occurrence of diarrhea and death (2, 3, 58, 93, 94, 98). The evidence for the protective capacity of most antigens is not conclusive, because immunizing preparations were often whole bacterial cells that shared more than one antigen with the challenge strain or because the antibody response to all potentially protective antigens in the vaccine was not measured. The most conclusive evidence has come from trials using biochemically pure fimbrial antigens for vaccination. The results of these studies are summarized below.

*Somatic Antigens.* There is no clear evidence of the protective ability of somatic antigens in vaccines against ETEC in calves. Vaccination with one strain of *E. coli* that shared only the somatic antigen with the challenge strain did not provide protection (94). However, results were inconclusive because the vaccine did not stimulate an increase of somatic antibody titers greater than those in unvaccinated control animals. Similarly, a whole cell bacterin prepared from autoclaved ETEC that destroyed the capsular and fimbrial antigens, but not the somatic antigen, also failed to protect. Because antibody titers were not measured in this trial, immunogenicity of the somatic antigen was not confirmed, and, hence, its protective capacity could not be determined (94).

*Capsular Antigens.* There is inconclusive evidence that capsular antigens are protective. There is only one report in which cows were vaccinated with purified capsular material (93). Results of this trial did not demonstrate a significant difference in the proportion of calves that were protected against experimental challenge between vaccinated (7/9) and unvaccinated control groups (4/9) ( $\chi^2 = 2.10, P > .05$ ) or in the proportion of protected calves nursing dams with high ( $> 6$ ) colostral anticapsular antibody titers (5/5) as compared with low ( $\leq 6$ ) antibody titers (2/4) ( $\chi^2 = 2.80, P > .05$ ). The trend toward protection might have been significant if this trial had included a larger number of

animals or a challenge strain that was more virulent for calves in the unvaccinated control group. This is suggested by combining the above results with those from a larger number of animals vaccinated with whole cell bacterins. The combined results show a significant correlation between high anticapsular antibody titers in colostrum and protection. However, these collective results are not definitive, because many of the animals that had high anticapsular antibody titers also had high anti-K99 antibody titers (93). Two other studies used whole cell bacterins that provided protection (94, 151). However, because the bacterins stimulated antibody formation to both capsular and somatic antigens, the protective capacity of the capsular component alone could not be evaluated clearly. In addition, the possible contribution of K99 and F41 fimbriae to protection was not eliminated. Attempts to confirm protective properties of capsular antigens by purified K30 antigen were unsuccessful because the purified antigen was poorly immunogenic even when coupled to sheep red blood cells and combined with adjuvant (Acres, unpublished data). However, recent work in swine using a whole cell bacterin containing a strain of ETEC, which shared only the capsular antigen with the challenge strain, demonstrated protection (84). The apparent ancillary role of capsules in colonization also suggests that they may contribute to protection, but this still remains to be confirmed. Currently there are at least two *E. coli* whole cell bacterins (Coligen, Ft. Dodge Laboratories, Ft. Dodge, IA and Coli-4, Franklin Labs. Ltd., Denver, CO) on the market that contain three capsular (K) antigens selected because they are among the most common on ETEC isolated in North America (66, 96). However, many other K antigens also occur (Table 1), and it would be impractical to incorporate them all into vaccines. These bacterins also contain fimbrial antigens, and the relative contribution of the capsular and fimbrial antigens to protection is not known.

*Fimbrial Antigens.* The most conclusive evidence for protection has been obtained with fimbrial antigens. These studies have been aided considerably because purified K99 (56) and F41 (27) antigens are highly immunogenic and sufficient material was available for vaccination trials. Acres et al. (3) showed that

calves nursing cows vaccinated prior to calving with purified K99 antigen were protected, whereas calves from unvaccinated cows were not protected. Protection correlated with K99 antibodies in colostrum even though the challenge strain (B44) carried both K99 and F41 attachment factors (3). A variety of different types of preparations including purified (3) and partially purified K99 (91, 98), K99<sup>+</sup> *E. coli* minicells (3), and whole cell bacterins (2, 24, 51, 95) have been used to induce K99 immunity. These preparations have protected calves against experimental challenge with a variety of different serotypes of ETEC including 08:K85:K99:H- (2), 09:K30:K99:F41:H- (2, 3), 09:K35:K99:H- (95), and 0101:K99 (98), which suggests that they will protect against most known wild-type bovine ETEC. Less than 3 to 4% of dairy cows have natural antibodies to K99 antigen (32, 64, 77, 128), and vaccination with K99-containing vaccines has been useful in problem herds. Vaccination with purified F41 antigen also protected against challenge with strain B44 although the degree of protection was not as complete as that with K99 antigen (Acres and de Graaf, unpublished data).

Additional evidence for the protective capacity of K99 antigens has been provided by trials using a monoclonal antibody (127). Under experimental conditions calves were protected against fatal ETEC when fed as little as 1.0 ml of a K99 monoclonal antibody produced in mice. If similar protection can be demonstrated under field conditions, the use of monoclonal antibodies may provide a convenient method of preventing diarrhea as well as other diseases of newborn calves.

Studies also have explored the immunizing potential of STa, another universal antigen produced by calf ETEC. In its native form, STa is nonantigenic, and attempts to induce antibody formation with crude preparations failed (48, 133). However, several investigators successfully raised neutralizing antibodies against STa by using purified (5, 41, 44, 62, 65) or biochemically synthesized toxin (63) coupled to protein carriers. The ability of the antibody to protect against purified STa or STa-producing ETEC (Figure 9F) was demonstrated in infant mice (5, 41, 65) and in ligated intestinal segments of rats (62, 63). However, neither of these two test systems mimics the natural

pathogenesis of ETEC infections; hence, confirmation that passive antibodies will protect against oral challenge still is needed. One attempt to confirm the ability of a STa-protein carrier complex to protect piglets nursing vaccinated sows was unsuccessful, perhaps because the immunizing preparation stimulated formation of relatively little neutralizing antibody in colostrum (81). There are not any reports of STa being used to vaccinate cows. Hence, STa-containing preparations have potential for use as vaccines; however, additional studies are required for confirmation.

*Flagellar Antigens.* The protective capacity of flagellar (H) antigens has not been explored. However, they likely will not be important immunizing agents because many ETEC are nonmotile and there is considerable variability of the flagellar antigens of motile types.

#### Mechanisms of Antibody-Mediated Protection

Antifimbrial antibodies are thought to prevent diarrhea by interfering with attachment of ETEC to the intestinal mucosa (Figure 9E) (2, 79, 91). It is likely that antibodies coat the binding sites on the pili and thereby inhibit attachment to specific receptor sites on the brush border (91). However, this has not been proved, and opsonization, agglutination, steric hindrance, or changes of bacterial surface charge are other possible mechanisms of protection. There is also recent *in vitro* evidence that fimbrial antibody may reverse adhesion, which suggests that they also may be useful for treatment as well as prevention of diarrhea (75). A demonstration of the ability of K99 antibody to bind to the pilus appears in Figure 3.

Other specific mechanisms that potentially could prevent ETEC infection include the bactericidal and bacteriostatic effects of antibodies (Figure 9B). However, the mechanisms by which antibodies mediate bacterial inhibition in the intestinal lumen are speculative and have not been demonstrated clearly. One possible mechanism is for antibodies to promote phagocytosis (7). During ETEC infections large numbers of potentially phagocytic cells, mainly neutrophils, migrate into lumen of the small intestine through the dome area of Peyer's patches (9, 10). These cells appear to be capable to phagocytosing ETEC (7); however, it is not known if complement and antibody are essential

mediators of this process as they are in other body systems (55). Normally, fixation of complement to bacterial cells is a prerequisite to phagocytosis. Bacterial capsules can block complement fixation thereby preventing phagocytosis. This antiphagocytic effect of capsules can be overcome by antibody against the capsular (K) or, in some cases, the somatic (O) antigen of the strain involved (55, 144). Therefore, antibodies may promote phagocytosis in the intestine, particularly when encapsulated strains of ETEC are involved. The ETEC also may be inactivated or destroyed by enzymes and antibacterial substances, such as lactoferrin, released by disintegrating neutrophils that have migrated through the wall of the intestine (10). Also, Reiter observed that lactoferrin alone inhibited growth of an encapsulated strain of ETEC but that inhibition increased markedly when antibody and complement were present also (115). The specificity of antibodies that supplement lactoferrin activity is unknown and does not appear to be associated with the classical O, K, or H antigens of *E. coli* (11). There is a suggestion that the antibody may act by preventing production of enterobactin (enterochelin), which could prevent bacteria from sequestering enough iron to support maximum growth (11).

Another potential immune mechanism against ETEC is antibody-induced elimination of virulence determinants. Linggood and others (69, 70) demonstrated that the K88 antigen was eliminated irreversibly from porcine ETEC by exposure to antiserum from vaccinated sows. Loss of K88 antigen was apparently due to the loss or "curing" of the plasmid that carries the gene encoding for fimbrial production. Because K88 is an attachment factor for piglets, "cured" bacteria were unable to colonize the small intestine or to cause diarrhea (Figure 9G). Neither K88 nor O-antigen specific antibodies caused this phenomenon, and antibody against an undefined bacterial surface antigen was thought to be responsible (69). A similar mechanism also could be active against ETEC in calves particularly when whole cell bacterins are used for immunization. If so, this mechanism could help not only to protect individual animals but also to reduce significantly environmental contamination with virulent ETEC.

There are now many ETEC vaccines used in many countries around the world. Most of these are whole cell bacterins formulated to provide high antifimbrial antibody that prevent diarrhea by blocking bacterial adherence. Some also contain capsular (K) antigens; however, as was pointed out above, their contribution to protection remains unclear. A potential advantage of whole cell bacterins and crude cell lysates or extracts is that they also may contain antigens that invoke other protective antibodies such as those responsible for plasmid curing or for interfering with enterobactin-mediated iron transfer to bacteria. New vaccines composed only of purified or synthetic fimbriae soon may be available. Comparative studies have not been done; however, a potential disadvantage of purified preparations may be that they will stimulate only antifimbrial immunity and not some of the other potentially protective mechanisms discussed above. Hence, the spectrum of protection provided by these preparations may not be as broad as that provided by whole cell bacterins, and this could be a disadvantage if "antigenic drift" in ETEC occurs in the response to vaccination pressure.

Various routes of inoculation have been used to stimulate immunity to ETEC. Most commercially available vaccines are administered either subcutaneously or intramuscularly and stimulate formation of serum antibodies that are transferred to, and concentrated in, the colostrum before parturition. The antibodies are transferred passively to the calf after birth and act locally within the small intestine to prevent ETEC infection. The immunoglobulin class in which the protective colostrum antibodies occur has not been determined; however, based on our general knowledge of lacteal immunity in the bovine, the majority of the protective antibodies are probably predominantly IgG and IgM. Protective antibody titers are provided during the colostrum period but decline rapidly and are probably negligible by 5 days postpartum (51, Acres, unpublished data). Oral vaccination of cows with *E. coli* antigens also was tried as a method of stimulating formation of K99 antibodies in postcolostral milk; however, this was not successful with either killed or live ETEC (83). In utero inoculation of the fetus to stimulate development of active

immunity before birth also has been tried experimentally (18, 102, 152). Inoculations were by nonsurgical insertion of a needle through the body wall and uterus into the amniotic fluid. Killed antigens deposited into the amniotic fluid were ingested by the fetus and stimulated formation of systemic and local immunity. Disadvantages of this method include the occurrence of a small percentage of abortions or still births, the need to accurately the injection position, and the need to estimate the breeding date so that immunization is during the final few weeks of gestation (18, 102, 152). Nonetheless, if some of these practical problems eventually can be overcome, in utero vaccination may provide a method of protecting calves against a variety of common diseases including enteric colibacillosis, colisepticemia (18, 42, 102), and viral diarrheas (100).

#### USE OF *Escherichia coli* VACCINES

Based on the studies described, several commercially available vaccines have been developed and are being used in the field with success (2, 24, 51, 95). These vaccines appear to be safe and efficacious and do not cause endotoxin or anaphylactic reactions, which were a problem with earlier generations of bacterins that contained *E. coli* or mixed Gram-negative bacteria. The quantity of K99 antigen, and probably other important antigens expressed by *E. coli* grown in artificial systems, depends on a variety of cultural conditions (28, 39, 57, 58). Therefore, *E. coli* cultures used for vaccine production must be monitored to ensure that sufficient K99 antigen is produced and that the vaccines will satisfy potency standards (39). Hence, only vaccines produced by federally licensed manufacturers should be used. The immunization schedule recommended with most of these vaccines calls for two inoculations during the 1st yr of an immunization program. In subsequent years a single booster inoculation within a few weeks of calving is recommended (51). Because the incidence of ETEC infections is sporadic and difficult to predict, and because there are other causes of diarrhea that will not be prevented by *E. coli* vaccines, the main difficulty in using this type of vaccine is in identifying those herds in which vaccination will be of economic benefit.

In early herds at least four criteria should be considered before vaccination. They are: 1) Occurrence of acute profuse watery diarrhea in calves under 4 or 5 days of age. Because calves do not appear to be susceptible to K99<sup>+</sup> ETEC after the first few days of life and because the concentration on antibodies in lacteal secretions is low by 5 days even in vaccinated cows [(51), Acres, unpublished data], vaccination will be most useful in herds where young calves are affected.

2) Diarrhea is economically important for the herd. Vaccination must be cost-effective for producers and, hence, should be recommended only when the incidence, severity, or both of diarrhea make investment in a vaccination program worthwhile. The economics of using *E. coli* vaccines in dairy herds has not been studied. However, a benefit:cost ratio of 5.9 was achieved in a cow-calf operation in which calves born to first-calf heifers were prone to ETEC infection because of confinement. This meant that each dollar invested in the vaccine returned an additional \$5.96 at weaning (73).

3) Presence of ETEC in diarrheic calves should be confirmed by laboratory diagnosis. In addition to ETEC there are many other causes of diarrhea that *E. coli* vaccines will not prevent. Hence, laboratory confirmation of etiology is desirable.

4) The management system of the herd will ensure that immune colostrum is fed within 3 to 4 h of birth and will continue for several more feedings. This will guarantee that locally protective antibodies are continually in the small intestine during the time calves are susceptible to colonization by ETEC. Definitive studies have not been done, but it appears that calves that ingest 1 to 2 liters of immune colostrum within 4 to 6 h of birth will be protected in most circumstances (Acres, unpublished observations). However, to guarantee maximum protection against ETEC, colisepticemia, and other diseases, calves should consume a volume of colostrum equal to 10% of their body weight within the first 25 h. At least half of this amount should be received within the first 6 h.

The protective capacity of two different commercial *E. coli* vaccines (VICOGEN, Connaught Labs Ltd., Swiftwater, PA and Coli-4, Franklin Labs, Denver, CO) was compared in

a vaccination-challenge experiment (Acres, unpublished data). Both products protected calves against experimental challenge with ETEC strain B44; however, protective antibodies appeared to differ. VICOGEN stimulated formation of K99 and F41 antibodies, whereas Coli-4 stimulated formation of F41 and K30 but not K99 antibodies. Therefore, vaccines should not be used interchangeably if maximum antibody titers and, hence, protection are to be obtained. Hence, during the 1st yr of a vaccination program, the same vaccine should be used for primary and booster injections. During subsequent years the same vaccine should be used for the single booster injection.

In some dairy herds the incidence of enteric colibacillosis is sporadic, and producers are reluctant to invest in a full program of vaccination. In such cases, it is often prudent to recommend that a few high-producing cows be vaccinated and that their excess colostrum be stored frozen in 1- or 2-liter aliquots. Frozen colostrum will retain its protective capacity for 2 yr or more. Repeated freezing and thawing, and temperatures above 56°C destroy antibodies and should be avoided. When needed, frozen colostrum should be thawed at room temperature or in warm water (below 56°C). It can be used to feed calves that have been deprived of colostrum for some reason or to supplement normal colostrum intake when an outbreak of *E. coli* diarrhea occurs. In some countries, sterile, freeze-dried colostrum from vaccinated cows is sold commercially (146). A K99 monoclonal antibody (Genecol 99, Molecular Genetics Inc., Minnetonka, MN) is also available and provides another source of passive antibody which can be used to prevent fatal diarrhea caused by K99<sup>+</sup> ETEC (127).

The impact of widespread immunization on the virulence characteristics of the natural population of ETEC is unknown. In the face of vaccination pressure against antigens such as K99 fimbriae or specific capsular polysaccharides, bacteria may develop new mechanisms of colonizing the small intestine. A more likely possibility is that ETEC that already possess other fimbriae, such as F41 or Att 25, may become more common. There is the suggestion that this has occurred in swine herds in which vaccines containing K88 antigen have been used (143). A study over 4-yr showed that about

50% of ETEC from diarrheic piglets in vaccinated herds were K88<sup>+</sup> and the remaining 50% carried K99, 987P, or other unidentified attachment factors. In contrast, in piglets from unvaccinated herds, 90% of ETEC were K88<sup>+</sup> and the other 10% were K99 or 987P<sup>+</sup>. This suggests that vaccination may have caused a shift of ETEC population away from K88<sup>+</sup> types toward types carrying other fimbriae, but this could not be proven because vaccinated and unvaccinated herds represented two different populations, and other factors could explain the observed differences. Additional long-term studies of the ETEC population in herds before and after vaccination will help confirm this possibility. Nonetheless, it appears that immunization may change the dominant ETEC population, but the degree and the rate at which this will happen remains to be determined. Hence, on-going surveillance and characterization of the ETEC population is necessary, and additional antigens may have to be incorporated into vaccines from time to time if major changes are detected.

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