Attaching-effacing *Escherichia coli* Infections in Cattle

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Escherichia coli was first recognized as a cause of diarrhea and septicemia in calves more than 115 years ago.[1] Intestinal infection with *E coli* manifested principally by diarrhea is commonly known as enteric colibacillosis, in contrast to septicemic and enterotoxemic colibacillosis, which are characterized by systemic infection and per-acute collapse.[2,3] Diarrheagenic *E coli* are now broadly placed into 6 classes based on virulence mechanisms.[4,5] One of these classes, enterotoxigenic *E coli* (ETEC), is the most common cause of diarrhea in beef and dairy calves in the first 4 days of life.[3,6] ETEC are characterized principally by the production of fimbriae and entero-toxins, with strains producing K99 (now called F5) fimbria and heat-stable entero-toxin-a (STa) as the main ones causing disease in calves.[3–6] Two other diarrheagenic classes, namely enterohemorrhagic *E coli* (EHEC) and enteropatho-genic *E coli* (EPEC), are important causes of disease in human beings, but less well substantiated causes of diarrhea in calves.[3–6]

E coli strains that cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans, express high levels of Shiga toxin (Stx), cause attaching-effacing (A/E) lesions in intestinal epithelial cells, and possess a specific 60-MDa EHEC plasmid are known as EHEC.[7] Hence, using this original definition, the causation of human illness (HC or HUS) was an obligatory criterion for identification of an isolate as EHEC.[7] One feature EHEC and EPEC have in common is the causation of intestinal epithelial lesions known as attaching and effacing (A/E).[4,5,8] Attaching-effacing *E coli* (AEEC) is a designation for those *E coli* strains known to cause A/E lesions or at least carry the genes for this trait, and therefore include organisms that fall into either the EHEC or EPEC classes.[4,5,8] A distinction between EHEC and EPEC is that the former produce Stx, whereas the latter do not.[4,5] *E coli* strains that produce or carry genes for the production of Stx (also known as Verotoxin or Verocytotoxin [VT], and formerly

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known as Shigalike toxin [SLT]) have been called Shiga toxin–producing *E coli* (STEC) or Verotoxin-producing *E coli* (VTEC).[4,5,9–13] Mainil and Daube[12] proposed that all *E coli* strains producing or carrying the genes for Stx but not A/E should be called STEC or VTEC, whereas those positive for both should be called EHEC. Unless stated otherwise, this nomenclature is adopted for use in this article. In addition, the term Stx is used instead of SLT or VT, and STEC instead of VTEC.

Cattle are a major reservoir of STEC and EHEC, including the prototype of this class, *E coli* O157:H7, which was the first *E coli* serotype recognized to cause HC and HUS in humans.[13–19] Worldwide, *E coli* O157:H7 is the EHEC serotype most often associated with causation of HUS.[20] EPEC and EHEC strains can belong to more than 1000 O:H serotypes[21]; there are more than 435 serotypes of STEC,[13,21,22] and more than 120 O serogroups of EHEC and STEC[12] have been recovered from cattle. Because cattle are carriers of many different serotypes of EHEC, much emphasis has been placed on the public health and food safety concerns associated with the fecal shedding of these organisms. However, much less emphasis has been given to their roles as diarrheagenic pathogens of cattle. Mainil and Daube[12] noted that certain subgroups are pathogens of cattle; in their review, they indicated that the most important ones causing diarrheal disease in calves were O5:H⁻, O26:H⁻, O26:H11, O111:H⁻, and O118:H6 . In contrast, in a more recent review article on the pathophysiology of calf diarrhea it was stated that the claim that AEEC are pathogens of calves is questionable.[6] The goal of the present article is to address the question of pathogenicity, with a review that focuses on the results of studies of natural and experimental infections with these organisms. The authors' conclusion is that there is overwhelming evidence that many different serogroups of AEEC are diarrheagenic pathogens of calves.

THE ATTACHING-EFFACING LESION

The first report of A/E lesions in any species was in 1969 by Staley and colleagues[23] in a study involving newborn gnotobiotic piglets inoculated with *E coli* O55:B5:H7. The purpose of the study was to demonstrate the "ultramicroscopic sequence of the attachment and penetration of *E coli* into ileal epithelial cells of the neonatal pig." The origin of the *E coli* strain was not stated, but O55:H7 is a classic EPEC of humans, and thought to be the evolutionary precursor of *E coli* O157:H7.[24] The ultrastructural lesions described in the article by Staley and colleagues[23] were bacterial attachment to enterocytes with degeneration and exfoliation of microvilli. These investigators also were particularly interested in bacterial uptake or phagocytosis by enterocytes of the newborn piglet, and this process was extensively described. The existence of different diarrheagenic classes, the importance of ETEC in the causation of porcine enteric colibacillosis, and the association of ETEC with the intestinal epithelium were not known at the time of this publication, nor was it known that pathogenic *E coli* strains might vary in their attachment mechanisms. Ten years later, Moon and colleagues[25] reported that different *E coli* strains vary in the mechanisms they use to associate with the intestinal epithelium. This study contrasted the rather loose adherence of ETEC mediated by pili with that of the *E coli* that tightly attach.[25] At this time, *E coli* that attach had already been described to occur in rabbits.[26] In their article, Moon and colleagues[25] showed a photomicrograph of bacteria attached to the surface of absorptive enterocytes in a typical A/E pattern in the colon of a calf. The investigators hypothesized that these organisms were *E coli*, and stated this could be evidence that a new enteropathogenic type of *E coli*, one that is nonenterotoxigenic and noninvasive, was emerging in cattle and other animal species.

In 1982, Rothbaum and colleagues[27] described the presence of ''enterocyte-adherent *E coli*'' in biopsies of the jejunum, rectum, or both in human infants with protracted diarrhea. The ultrastructural appearance of the association between bacteria and enterocyte was that of the A/E lesion, and the causative organisms were *E coli* O119:B14, a classic EPEC serotype.[27] In 1983, Moon and colleagues[8] coined the term ''attaching and effacing'' for the lesion characterized by intimate bacterial attachment and effacement of microvilli, as originally described by Staley and colleagues[23] and later reported to occur in rabbits and humans.[26,27] These investigators also coined the term ''attaching-effacing *E coli* (AEEC)'' for those *E coli* organisms that cause A/E lesions.[8]

EVIDENCE OF AEEC AS CATTLE PATHOGENS BASED ON STUDIES OF NATURAL AND EXPERIMENTAL DISEASE
Disease in Calves Caused by E coli O5, O26, O111, O118, and O145 Infection

A series of 3 articles describing a dysentery syndrome that occurred in 8- to 21-day-old calves at a research farm in England from the autumn of 1981 until the spring of 1983 were the first reports to confirm the presence of AEEC in cattle.[28–30] On this farm, the syndrome affected calves derived from a herd of Friesian cows that, when 3 days old, had been allocated to an individual pen. During the stated period, 12 of approximately 400 calves that had been allocated to the pen developed dysentery. Dysentery was first seen in calves at 8 to 21 days of age (mean 15 days), and characterized by the passage of copious bright red blood in the feces. Feces became liquid in all dysenteric calves concurrent with or 1 to 5 days before the onset of dysentery. Most calves had no signs of systemic illness at the onset of dysentery, nor pyrexia, and initially, normal appetite was maintained; however, signs of dehydration, dullness, anorexia, reluctance to move, weight loss, and death occurred in some cases. In addition, persistent grinding of the teeth, abdominal distention with pain on palpation, and a noticeable smell of necrotic tissue were recorded in some calves.

In the first attempt to identify the cause of the dysentery syndrome, Chanter and colleagues[28,29] administered intestinal contents and feces from affected calves, or microorganisms isolated from them, to 1-day-old gnotobiotic calves. Inoculated calves passed normal feces for 2 to 4 days after inoculation, and for the next 4 to 7 days (until euthanasia), they passed mucoid, liquid feces containing many small clots of fresh blood. At necropsy, the walls of the colon and rectum were thickened, and there was patchy reddening on the longitudinal folds of the mucosae of the cecum, colon, and rectum. Petechial hemorrhages were numerous and occasionally larger hemorrhages were seen, with clotted blood adherent to the mucosal surface. In some areas, there was an adherent layer of mucus stained with intestinal contents. The production of dysentery and colonic lesions was strongly associated with colonization by an atypical form of *E coli*, designated S102-9. On quantitative bacterial culture, the mucosae of the ileum, colon and rectum contained as many as 10^6, 10^{10}, and 10^9 *E coli*, respectively, and these numbers were approximately 4 times higher than those in the gut lumen. The calves were bacteremic, but bacterial counts in the blood were less than that typically seen with septicemia-inducing *E coli* strains. The majority of biochemical reactions of this organism were typical of *E coli*, but it produced urease, and was anaerogenic and nonmotile; its serotype was O5:K$^-$:H$^-$. Colonies on MacConkey agar had a characteristic red center and a clear outer zone; the surrounding medium did not exhibit a red precipitate. Strain S102-9 was identified as *E coli*, in particular by its ability to produce acid in MacConkey broth at 44°C and indole at 44°C.

In a separate report from the same study, the etiology was also addressed, but this report focused more on the pathologic changes of the dysentery syndrome.[29] Lesions in calves with the naturally and experimentally produced syndrome were limited to the intestinal tract, although gross lesions in the large intestines of gnotobiotic and farm calves varied.[29] Ceca appeared normal on gross examination except in one farm calf in which it was hyperemic and had undergone intussusception into the colon. Colonic lesions varied from mild patchy reddening with petechial hemorrhages, adherent mucus, and blood clots. No gross lesions were seen in the rectum in any of the calves with the exception of hyperemia of superficial extremities of the longitudinal folds. In the ileum in 4 of 5 farm calves and a gnotobiotic calf, villi were atrophic, fused, and covered by cuboidal or flattened enterocytes. Bacteria were seen in association with irregularly arranged and exfoliated enterocytes in one gnotobiote. There was an increase in the numbers of neutrophils in the lamina propria of the villi of one farm calf, and in the gnotobiote with adherent bacteria. In all calves, cecal lesions were mild; foci of adherent bacteria were associated with clumps of irregularly arranged and exfoliated enterocytes, and increased numbers of neutrophils were seen in the lamina propria. Neutrophils were seen rarely in the crypts, or on the luminal surface.

In all calves, lesions were most severe in the colon and rectum, where there was hyperemia of mucosal capillaries with occasional petechial hemorrhages on the luminal surface. Bacteria appeared to be adherent to the mucosal surface between the mouths of the crypts and sometimes into the crypts for approximately 10% of their length. Adherent bacteria could be seen in sections stained with hematoxylin and eosin, but were more clearly visible in sections stained with Giemsa or in semithin sections of tissue embedded in epoxy resin and stained with toluidine blue. The epithelial surfaces containing adherent bacteria were irregular, due to enterocyte degeneration and exfoliation. The mucosa was often edematous and infiltrated with neutrophils, which also had entered some crypt lumens and exuded onto the luminal surface. The mucosal surface also contained mucus, exfoliated enterocytes, and erythrocytes.

Typical A/E lesions were seen in the colon in farm and gnotobiotic calves by scanning and transmission electron microscopy. Under scanning electron microscopy, the distribution of bacilli on the surfaces of enterocytes was sometimes restricted to intercryptal regions, but in some cases covered the entire surface. Microvilli of infected enterocytes were absent, or were abnormal in orientation or length, either shortened or elongated. Exfoliated enterocytes bearing these changes were also seen on infected surfaces. Bacteria were noted by transmission electron microscopy to be closely associated with the enterocyte cell membrane. At sites of bacterial adherence the microvilli were effaced, and other microvilli not containing adherent bacteria were frequently distorted and disorientated. At the points of bacterial attachment, the enterocyte cytoplasm typically was cup-shaped or arranged as a pedestal.

In histologic sections stained by an immunoperoxidase technique, several enteropathogens in a gnotobiotic calf inoculated with feces were detected, namely rotavirus, coronavirus, E coli S102-9, and Campylobacter spp. In contrast, the 2 gnotobiotic calves inoculated only with E coli (S102-9), confirmed that S102-9 was detectable in sections using the immunoperoxidase method; these calves were not tested for other enteric pathogens. In 3 of the 5 dysenteric farm calves, the mucosal surface of the large intestine contained surface-attached bacteria that stained positively with antiserum to E coli (S102-9). In the other 2 farm calves with dysentery, typical lesions were seen in the large intestine, but bacteria adherent to the mucosal surface did not stain positive with antisera to S102-9.

In a second study, the dysentery syndrome was experimentally induced in 5 4-day-old colostrum-fed calves after inoculation with S102-9.[30] The clinical, microbiological, and pathologic features were essentially the same as those described in the first 2 reports.[28,29] However, in this study immunity and age resistance were investigated; dysentery was not seen following a second challenge in calves that had recovered, nor was it seen in an age-matched calf at 24 days of age, nor in a 51-day-old calf. S102-9 did not produce heat-stable enterotoxin, but did produce a toxin cytopathic for Vero and HeLa cells (ie, Stx). In addition, the investigators also conducted a survey of field cases of calf diarrhea in southern England during the winters of 1981 to 1982 and 1982 to 1983.[30] Of 659 lactose-fermenting bacterial isolates, including 373 from calves with diarrhea, 4 had an atypical colony morphology on MacConkey agar indistinguishable from that of *E coli* S102-9, were anaerogenic and produced urease. Based on other tests, these isolates were identified as *E coli*. The 4 isolates originated from different farms and 3 were isolated from calves with diarrhea. Coronavirus was isolated from one of these calves and *Salmonella typhimurium* from another; the third isolate from a normal calf was designated 6/193, and the fourth from a calf with diarrhea in which other enteropathogens were not detected was designated 37/1. Preliminary serotyping of 37/1 and 6/193 revealed they were O5. Therefore, *E coli* phenotypically indistinguishable from S102-9 was detected in diarrheic calves from other farms in southern England; however, because only 4 of 659 isolates were identical to S102-9, this organism was concluded not to be an important cause of enteric disease in the survey. It was noted that one isolate with these properties had previously been associated with diarrhea in a calf in France.[29,31]

An *E coli* phenotypically very similar to S102-9 was isolated in the United States from a 2-day-old beef calf in Minnesota with nondysenteric diarrhea.[32] The clinical case isolate, 84-5406, was a urease-positive O5:K4:H⁻ *E coli* that produced Stx, but did not produce enterotoxins and was noninvasive; its colony morphology on MacConkey agar was unremarkable. Isolate 84-5406 was sensitive to ampicillin, cephalothin, chloramphenicol, furazolidone, gentamycin, kanamycin, polymyxin B, spectinomycin, sulfasoxazole, and trimethoprim-sulfamethoxazole, and was resistant to penicillin and tetracycline. The calf from which this organism was isolated was co-infected with rotavirus and coronavirus, and had evidence of villous atrophy in the small intestine and diffuse colonization of the colonic epithelium by bacteria typical of AEEC. A conventional colostrum-deprived lamb was inoculated with a pure culture of 84-5406, and this animal developed bloody diarrhea and died within 5 days after inoculation. Histologically, the colon of the lamb was found to be diffusely colonized with bacteria, and the inoculum strain was isolated from this tissue. Three 1-day-old gnotobiotic calves subsequently were inoculated with 84-5406; the calves were checked at 12-hour intervals post inoculation (PI) for anorexia, depression, fever, diarrhea, and the presence of mucus or blood in the feces. The calves developed a mild fever (0.5°C elevation) at 36 hours PI, which peaked (0.9°C increase) at 48 hours PI and fluctuated slightly thereafter for 1 to 3 more days until it returned to normal. The calves became depressed when the temperatures reached 40.0°C, but none became anorectic. Two of the calves developed diarrhea by 36 hours PI. The diarrheic feces were loose, dark green, and mucoid, and by 60 hours PI the feces of one diarrheic calf contained frank blood. The diarrhea in the calf that did not become dysenteric lasted only 24 hours.

At necropsy, hyperemia of the longitudinal folds of the rectum was noted in both the 84-5406 and control calves, and microscopic lesions were limited to the intestines of the former.[32] Bacterial colonization was seen multifocally in the ileum, and diffusely in the large intestine. Bacteria were noted to be closely attached to the surfaces of

enterocytes, and many cells with attached bacteria had become necrotic and sloughed into the intestinal lumen. Loss of enterocytes from the surfaces of villi had resulted in villous atrophy in the ileum (**Fig. 1**). Bacterial colonization and enterocyte sloughing in the cecum, colon, and rectum were diffuse (**Fig. 2**), and associated with minimal acute multifocal inflammatory reaction characterized by edema and neutrophilic infiltration in the lamina propria. Bacteria colonizing the intestines were noted to have stained positive with anti-O5 serum when observed by immunofluorescence microscopy. In contrast, the epithelium of the colon and rectum of gnotobiotic calves inoculated with sterile broth is smooth with intact enterocyte junctions and densely packed microvilli (**Figs. 3** and **4**). Under scanning electron microscopy, the colon and rectum in gnotobiotic calves inoculated with 84-5406 were noted to have diffuse AEEC bacterial colonization, causing epithelial sloughing and irregularity of the mucosal surface (**Fig. 5**). On higher magnification, microvillous effacement at sites of intimate bacterial attachment, that is, classic A/E lesions were seen both by scanning (**Fig. 6**) and transmission electron microscopy (**Fig. 7**). Extensive AEEC infection of individual enterocytes resulted in death and sloughing of these cells; this is shown by transmission electron microscopy in a calf infected with 84-5406 (**Fig. 8**). A diagnostic case of natural disease in a calf infected with an unidentified serotype of AEEC is shown histologically in **Fig. 9**. In the diagnostic case shown in **Fig. 9**, bacterial colonization was extensive and had caused death and exfoliation of colonic mucosal epithelial cells. Persistence of bacterial colonization and epithelial sloughing resulted in AEEC bacteria brought into close proximity to the underlying basement membrane.

Fig. 1. Light micrograph of ileum from gnotobiotic calf inoculated with *E coli* O5:H⁻ strain 84-5406. There is multifocal enterocyte necrosis and detachment associated with microcolonies of bacteria (*inset*) attached to apical cell membranes. Loss of enterocytes has resulted in villous atrophy (as evidenced by a villus:crypt ratio of 1 in this affected villus). Bar = 100 μm. Inset shows detail of bacterial microcolonies (bar = 5 μm). (*From* Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 1986;53(2):339–46; with permission. Copyright © 1986 American Society for Microbiology.)

Fig. 2. Light micrograph of rectum from gnotobiotic calf inoculated with *E coli* O5:H⁻ strain 84-5406. Bacterial colonization is diffuse and extensive, with diffuse enterocyte necrosis and detachment (*arrows*). Colonization is mainly on the mucosal surface in intercryptal regions (denoted in scanning electron micrographs as ridges) and crypt openings; bacterial colonization deep into the crypts is not seen. Bar = 100 μm. Inset shows detail of bacterial microcolonies (bar = 5 μm). (*From* Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 1986;53(2):339–46; with permission. Copyright © 1986 American Society for Microbiology.)
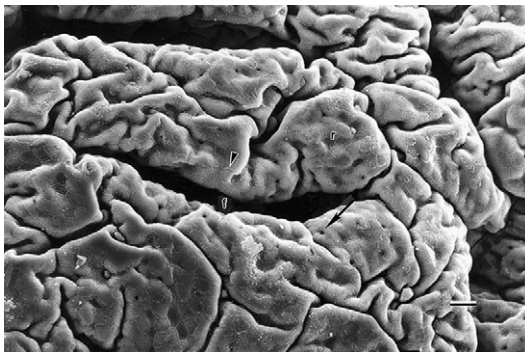

Fig. 3. Scanning electron photomicrograph of colon of a gnotobiotic calf that was sham-inoculated with sterile trypticase soy broth when 24 hours old and euthanized 7 days later. The mucosal surface is smooth and completely devoid of microbial flora. The mucosal surface is arranged in ridges (r) separated by furrows (f), into which crypts (not seen at this magnification) open. Small circular holes on the mucosal surface (*arrow*) demarcate pits created by goblet cells that have discharged their mucus. Hexagonal outlines of enterocytes (*arrowhead*) are evident at this magnification (original negative magnification ×500; bar = 20 μm).
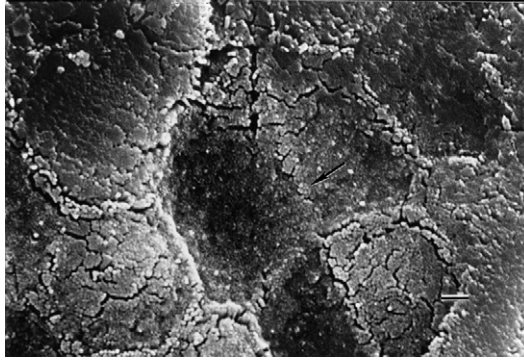
Fig. 4. Higher magnification scanning electron photomicrograph of colon of sham-inoculated gnotobiotic calf. Microvilli are seen as a dense mat on the surfaces of enterocytes. Outlines of enterocytes (*arrow*) appear as slight ridges (original negative magnification ×5000; bar = 1 μm).

Loss of epithelium had resulted in enteric mucosal atrophy and hemorrhage. Subsequent to the reports of S102-9 and 84-5406, other studies of natural and experimental AEEC infections in calves were reported in different countries.

Pospischil and colleagues[33] described naturally occurring AEEC infections in a retrospective study of the intestines of 3 calves in West Germany. The calves had diarrhea and catarrhal enteritis, and 2 were on a combined experimental rotavirus and ETEC study. Two of the calves were 7 days old and one was 23 days old. At necropsy, all 3 had catarrhal enteritis or gastroenteritis, and other complicating lesions. Histologically, there was diffuse atrophy and focal fusion affecting villi in the mid- and distal jejunum, and ileum. A/E bacteria were detected in the ileum, cecum and colon of all 3 calves, but were not seen in the proximal jejunum, mid-jejunum, or distal jejunum. Jejunal, ileal, and colonic epithelium of one calf contained numerous *Cryptosporidium*. A heavy layer of bacteria was seen in the cecum and colon, and on

Fig. 5. Scanning electron photomicrograph of colon from calf 7 days after oral inoculation with strain 84-5406. The mucosal surface is roughened due to rounding and detachment of enterocytes, and diffuse bacterial attachment to the apical surfaces of these cells. Microvilli are visible; an individual enterocyte that has undergone rounding and is in the process of detachment is denoted by an arrow. Ridges (r) have atrophied due to cell loss, and in this figure are approximately one-half the width of that of the control calf. Furrows (f) demarcate boundaries of ridges, as in the control (original negative magnification ×500; bar = 20 μm).
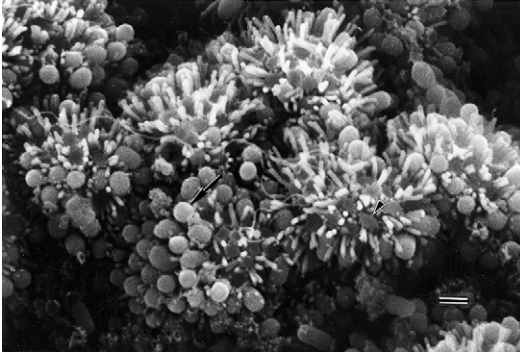
Fig. 6. Higher magnification scanning electron photomicrograph of colon shown in previous figure. Strain 84-5406 bacteria (*arrow*) cover the apical cell membranes of enterocytes. Cuplike or pedestal-like distortions of the apical cell membranes (*arrowhead*) are present at sites were bacteria were detached during tissue processing. Microvilli between attached bacterial cells are prominent and elongated. Enterocytes are swollen and are in the process of detachment from the mucosal surface (original negative magnification ×5000; bar = 1 μm). (*From* Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 1986;53(2):339–46; with permission. Copyright © 1986 American Society for Microbiology.)

transmission electron microscopy, typical A/E lesions were seen. One *E coli* isolate from the colon of one calf, identified as O23:K⁻:NM, produced Stx.

Schoonderwoerd and colleagues[34] reported 2 natural cases of AEEC infection of 5-week-old veal calves with a Stx-producing *E coli* O111:NM isolate, and experimental reproduction of disease with the isolate in a colostrum-deprived calf. Natural infection was characterized by pseudomembranous ileitis, and mucohemorrhagic colitis and proctitis. The calves also had evidence of systemic infection with increased fluid
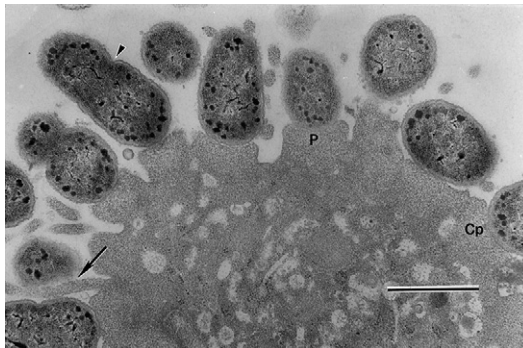
Fig. 7. Transmission electron photomicrograph of rectum from calf inoculated with strain 84-5406. The attachment of bacteria to this enterocyte results primarily in pedestal-like cell membrane evagination (P), with occasional cuplike invagination (Cp). Microvilli between sites of bacterial attachment are elongated (*arrow*). Some bacteria are in the process of binary fission (*arrowhead*). The cytoplasm lacks a discernible terminal web and contains numerous vacuoles (original negative magnification ×15,000; bar = 1 μm). (*From* Moxley RA, Francis DH. Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect Immun 1986;53(2):339–46; with permission. Copyright © 1986 American Society for Microbiology.)
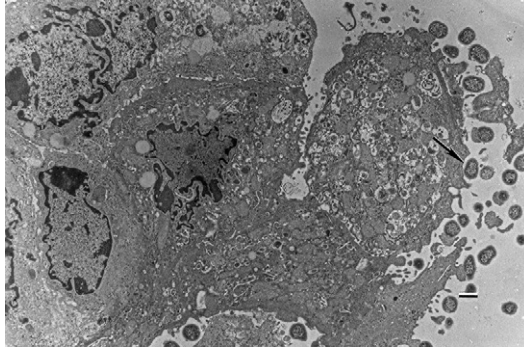
Fig. 8. Transmission electron photomicrograph of rectum from calf inoculated with strain 84-5406. An enterocyte containing many attached bacteria is necrotic, and in the process of sloughing from the mucosal surface. An individual attached bacterial cell is denoted by an arrow. Bacteria reside on pedestals, but bacterial release into the intestinal lumen is evident, some with bacteria still attached to fragmented remains of enterocytes (original negative magnification ×3000; bar = 1 μm).

and fibrin strands in some joints, a mild diffuse fibrinous peritonitis, swelling and hemorrhage in the kidneys, and enlargement of the mesenteric lymph nodes. Histologically, in the ileum there was villous atrophy in association with bacterial adherence, epithelial sloughing, fibrinosuppurative inflammation, and hemorrhage. Similar lesions were seen in the colon. Adherent bacteria in the ileum and colon stained positive with *E coli* O111 antiserum by immunohistochemistry. Cultures of the ileum and colon of both calves yielded a heavy growth of *E coli* O111:NM, which on further testing was shown to produce high levels of Stx. The *E coli* isolates were urease negative and unremarkable with regard to colony morphology. The isolates were resistant to
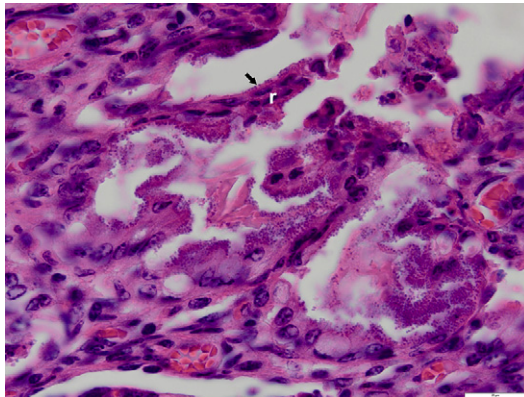
Fig. 9. Light micrograph of colon from a calf naturally infected with attaching-effacing *E coli*. AEEC had in this case had colonized both the small and large intestines. Bacterial proliferation has resulted in the formation of dense mats of organisms in intimate contact with the epithelium. Bacterial colonization extends to the depths of some crypts. Enterocytes with adherent bacteria have undergone atrophy. Sloughing of infected enterocytes has further resulted in atrophy of the ridges (r), with bacteria coming in close proximity to the basement membrane (*arrow*). Bar = 20 μm.

ampicillin, carbenicillin, sulfasoxazole, tetracycline, trimethoprim-sulfamethoxazole, and spectinomycin, and sensitive to chloramphenicol, gentamicin, neomycin, kanamycin, and nitrofurantoin. A 4-day-old colostrum-deprived Holstein bull calf that was negative for pathogens was inoculated orally with the *E coli* O111:NM isolate. The rectal temperature increased to 40°C on day 4 PI, and remained elevated until the next day when the calf was euthanized; the calf did not develop diarrhea during the 5-day PI period. At necropsy, the mesenteric lymph nodes were moderately enlarged, and the contents of the cecum and proximal colon were watery. Histologically, villous atrophy was seen in the duodenum, jejunum, and ileum. Bacterial adherence was detected only in the large intestine, more so in the colon than the cecum, and was associated with sloughing of mucosal epithelial cells and acute purulent inflammation. Adherent bacteria stained positive with anti-O111 serum by immunohistochemistry, and typical A/E lesions were detected in the large intestine by scanning and transmission electron microscopy. No evidence of bacterial invasion into the intestinal epithelium was detected.

Mainil and colleagues[35] tested 429 *E coli* isolates from calves younger than 1 month for the production of HeLa cell cytotoxins. The isolates came from calves that had enteric disease or systemic disease thought to have been caused by *E coli*. HeLa cell cytotoxic activity that was neutralizable with monoclonal antibodies to Stx1 or Stx2 were concluded to be due to those respective toxins; 4 isolates produced Stx1 and 1 produced Stx2. Four of the 5 isolates were typeable, and 1 each was found to be O26ab, O26, O22, and O111. Calves were experimentally inoculated with 2 of these isolates. The calves, when younger than 1 day, were allowed to suckle the dam, and then removed. Nine were inoculated when 5 to 10 days old; 6 calves were inoculated with isolate 1625 (O26:K⁻:H11, Stx1+), and the other 3 calves were inoculated with the nontypeable isolate, 211 (Rough:K?:H11, Stx2+). Isolate 1625 had been shown to cause A/E lesions in 4 of 6 inoculated rabbit loops, whereas isolate 211 did not induce A/E lesions in any of 6 inoculated rabbit loops. None of the calves inoculated with either isolate developed gross lesions in their alimentary tracts, and none developed diarrhea. Six of the calves were euthanized and necropsied, one from each strain inoculation group at 5, 7, and 9 days PI. The geometric mean viable *E coli* counts in the intestines for all calves combined were calculated. Bacterial counts in the intestines were 7×10^3 per 5-cm segment from the ileum, and 2×10^5 per 5-cm segment from the spiral colon. No microscopic lesions were seen in the intestinal tracts of any of the 3 calves inoculated with isolate 211, nor were they seen in the calf that was necropsied on day 9 after inoculation with strain 1625. However, spiral and pelvic colons from the 2 calves inoculated with isolate 1625 and examined at 5 or 7 days PI had a few focal lesions characteristic of those produced by AEEC. Lesions in the calves were qualitatively similar to those described in the calf from which strain 1625 was isolated originally, but were fewer in number and smaller in diameter. Presence of AEEC was confirmed by electron microscopy. Mainil and colleagues[35] concluded that increased Stx production and AEEC activity occur together in some *E coli* isolates from calves in the United States. However, the prevalence of such isolates in the collection studied was low. The bacteria apparently did not colonize well, and the A/E lesions induced in this study were not extensive enough to cause diarrhea. The AEEC lesions in the calf naturally infected with isolate 1625 were more numerous than those in the experimentally infected calves. Mainil and colleagues[35] noted that the isolate, which had been stored frozen for nearly 20 years before the study, might have lost virulence during storage, or the calves may have been immune or genetically resistant to the isolate.

Janke and colleagues[36,37] described natural cases of AEEC infections of calves presented to 2 veterinary diagnostic laboratories in South Dakota and Minnesota. Sixty cases of AEEC infection were detected from 59 farms in 7 states (South Dakota, Minnesota, Iowa, Nebraska, North Dakota, Wisconsin, and Michigan). Eighty-seven percent were dairy calves; 82% were Holstein or Holstein crossbreds. Diarrhea was the predominant clinical sign in calves with AEEC infection, with blood seen in the feces in 48% of the cases. However, 73% of the calves were infected concurrently with other enteric pathogens (cryptosporidia, rotavirus, coronavirus, ETEC, bovine viral diarrhea virus, and other coccidia). In 27% of the cases, AEEC was the only enteric pathogen identified. The age range of the calves was 2 days to 4 months, and the average age, excluding the 4-month-old animal, was 11.8 days. Eighty-eight percent of the calves were 2 to 21 days old; over half (51.7%) were 7 to 14 days old, and 36.6% were 2 to 6 or 15 to 21 days old (18.3% in each group). In 23.3% of the cases, extraintestinal lesions, such as pneumonia, arthritis, septicemia, and peritonitis, were detected. STEC were recovered from 31 of 46 calves; serotyping studies were conducted on 17 Stx-producing isolates. The predominant serotype was O111:NM; of 17 isolates, 9 (52.9%) were O111, 3 (17.6%) were O5:NM, 1 (5.9%) was O26:NM, and 4 (23.5%) were nontypeable. Fifteen of 60 calves (25%) at necropsy had grossly evident congestion and hemorrhage of the intestinal mucosa, and lesions were most pronounced in the large intestine. In 14 of 60 calves (23.3%), the feces were bloody. In the most severe cases, there was diffuse hyperemia and focal hemorrhage of the colonic mucosa, and the contents of the colon were blood-tinged with clots, necrotic debris, and mucus. In 2 calves, the cecum was hemorrhagic. Under light microscopy, A/E bacterial adherence to the intestinal mucosa was detected in the large intestine only in 34 of 60 calves (56.7%); in the small intestine only in 7 of 60 (11.7%); and in both locations in 19 of 60 (31.7%). Hence, A/E bacterial attachment was seen in the large intestine in 53 of 60 calves (88.3%). The investigators postulated that the reason for the preponderance of dairy calves in the study was because most were immunodeficient due to inadequate colostral intake.

Dorn and colleagues[38] characterized STEC isolates obtained during 1983 to 1989 from calves with diarrhea submitted as cases to diagnostic laboratories in South Dakota and Minnesota; an unspecified proportion of these isolates originated from previously published studies.[32,36,37] Thirty-six STEC isolates, each from a different calf, were identified for the study. These isolates came predominantly from calves designated as dairy or dairy-crossbred; only 4 of the isolates were from beef or beef-crossbred calves. One calf was 3 months old and all others of known age were less than 3 weeks old. Thirty-two of the 36 calves had bacteria colonizing the intestines and diarrhea, 3 had colonization with no observed diarrhea, and 1 had diarrhea but colonization was obscured by postmortem autolysis. Thirteen of the calves were observed by histopathology to have lesions of the colon. Twelve of the calves were observed to have bloody diarrhea. All 36 isolates fermented sorbitol within 24 hours. Twenty-one isolates were serogroup O111; 4 were O5, 2 were O26, 2 were O45, 1 was O69, 2 were O103, and 4 were nontypeable. The 4 O5:NM and 2 other isolates did not ferment raffinose. All but one isolate, 84-5406 (O5:NM),[32] hybridized with a probe for the 60-MDa EHEC plasmid. Thirty-two isolates hybridized with the stx_1 probe, 3 hybridized with both the stx_1 and stx_2 probes, and 1 (isolate 84-5406) hybridized with neither probe. The verotoxigenic activity of isolate 84-5406 was partially neutralized by monoclonal antibodies against Stx1.

At least 2 studies have provided evidence that non-Stx-producing AEEC are pathogens of calves and typically infect both the small and large intestines. Pearson and colleagues[39] reported the detection of AEEC in the small and large intestines of

a 3-week-old calf with yellow, watery diarrhea. The calf had no evidence of blood in the intestines or feces, and no pathogens other than the AEEC were isolated. AEEC were detected by transmission electron microscopy of specimens obtained surgically under general anesthesia. Histologically, AEEC were detected in the proximal jejunum, lower jejunum, ileum, cecum, and colon. The bacteria stained positively by an immunohisto-chemical procedure using antiserum raised against an *E coli* recovered from the calf; however, they did not stain using antiserum to S102-9, the O5:NM Stx1-producing AEEC previously reported.[28–30] Affected areas of small intestine colonized with the AEEC had sloughing of enterocytes with villous atrophy, villous fusion, and inflammation of the mucosa characterized by infiltration with neutrophils and plasma cells. Lesions in the small intestine were most severe in the ileum. AEEC bacterial adherence and epithelial sloughing were also seen in the cecum and colon. By electron micros-copy, typical A/E lesions and pedestal formation were seen in affected areas in the small and large intestines. The serotype of *E coli* isolated from this calf was not re-ported; however, it was a non-Stx-producer. Fischer and colleagues[40] detected a non-Stx-producing AEEC O26:NM isolate (7996-90) as the sole pathogen isolated from a 14-day-old Simmental calf with diarrhea. The isolate did not produce entero-toxins, Stx, or ETEC fimbriae, and was genotypically negative for the EPEC adherence factor (bundle forming pili), but it induced localized adherence in HEp-2 cells. In addi-tion, it induced A/E lesions in Caco-2 (human colonic carcinoma cells), rabbit intestinal loops, and large intestines of gnotobiotic piglets.[40] The lack of Stx production, coupled with localized adherence on HEp-2 cells and induction of A/E lesions, were taken as conclusive evidence that the isolate was an EPEC.

Wray and colleagues[41] inoculated colostrum-fed and colostrum-deprived neonatal calves 1 of 2 *E coli* isolates that produced either Stx1 or Stx2. The Stx1+ isolate A56 was O26:K60:H11, and the Stx2+ isolate A52 was O8:K85:H9. Five calves at the time of inoculation were 1 day old, and the remaining 4 were 2, 4, 16, or 17 days old. Only calves that were colostrum-deprived and 1 day old when inoculated developed A/E lesions or diarrhea. Both A56 and A52 induced A/E lesions in the small and large intes-tines in respective calves. Three of 4 colostrum-deprived calves developed anorexia, a slight elevation in rectal temperature (<1°C), and diarrhea with blood and mucus in their feces. At necropsy, they had hyperemia of the mucosa of the ileum and colon and enlargement of the mesenteric lymph nodes. Histologically, both A56 and A52 induced villous atrophy in the ileum secondary to bacterial colonization and epithelial cell sloughing. Typical A/E lesions were seen in the ileum and colon by transmission elec-tron microscopy with both strains. The investigators noted that this was the first report of a Stx2+ *E coli* being associated with disease in calves. However, what was perhaps more important was the demonstration of an apparently protective effect of age and colostrum in AEEC infections.

Stordeur and colleagues[42] isolated an *E coli* O118:H6 strain (340S89) from a 2-week-old Friesian calf that died of diarrhea. The strain was gene probe positive for in-timin and Stx1, and caused A/E lesions in rabbits. In an attempt to reproduce disease with the strain, 4 naturally born calves that were isolated immediately after birth, and then were washed, disinfected, and fed colostrum were used. The colostrum was demonstrated to lack agglutinating antibodies against strain 340S89. At 6 hours of age, 3 of the calves were inoculated orally with strain 340S89, and at 44 or 64 hours PI they were euthanized. One calf that was not inoculated was used as a control. The 3 calves inoculated with 340S89 developed mild hyperthermia and nonbloody diarrhea at 24 hours PI; the control calf did not develop diarrhea or other clinical signs of disease. Strain 340S89 was re-isolated from the feces of the 3 inoculated calves, but not from the control calf. The 3 inoculated calves developed A/E lesions in the

large intestine or both the small and large intestines, and a neutrophilic-lymphocytic enterocolitis.

Sandhu and Gyles[43] compared the pathologic effects of STEC that vary in their association with bovine and human disease. The pathogenicity of STEC serotypes associated with both dysentery in calves and HUS in humans (O5:H⁻, O26:H11, O111:H⁻, O113:H21) were compared with that of STEC O157:H7, which is associated with HUS in humans but less well documented disease in calves. The STEC were administered into ligated loops in the ileum and colon of 4 2- to 6-day-old calves. Strains of all serotypes tested except O113:H21, that is, O5:H⁻, O26:H11, O111:H⁻, O113:H21, and O157:H7, adhered focally to enterocytes and caused A/E lesions in both the ileum and colon. Acute neutrophilic inflammation was seen in inoculated loops, and these lesions were generally more severe in the ileum than the colon. Although the investigators did not discuss it, these results suggest that many different AEEC regardless of serotype are generally capable of causing A/E lesions in either the small or large intestine if present in high enough dosage, for example, enough to over-come immunity, and for long enough to make contact with mucosal enterocytes (eg, conditions present in a ligated gut loop).

Disease in Older Cattle Caused by AEEC Infection

There are at least 3 reports in the literature of older calves or adult cattle having entero-colitis and dysentery in association with AEEC infection. Janke and colleagues[36,37] described a case in a 4-month-old dairy calf in the Midwestern United States. However, the most remarkable cases were reports of fatal disease in a 19-month-old cow in Japan,[44] and another in an 8-month-old heifer in the United Kingdom.[45]

Wada and colleagues[44] reported that a 19-month-old Holstein cow manifested mu-cohemorrhagic diarrhea, anorexia, and depression after being out on a public pasture for approximately 2.5 weeks. This cow was shedding coccidial oocysts (300 per gram of feces) and was given sulfadimethoxine and Ringer solution intravenously; however, the aforementioned clinical signs continued, and the animal was euthanized. At necropsy, a large amount of bloody contents with mucus was present in the colon, and the mucosa contained petechial hemorrhages. The mesenteric lymph nodes were enlarged. Histologically, the mucosal epithelium in the colon was irregular and numerous gram-negative bacteria were notably adhered to the surfaces of entero-cytes. The lamina propria was hyperemic or hemorrhagic and contained a neutrophilic infiltrate. The intestinal crypts were dilated with mucus and the colonic lumen con-tained necrotic enterocytes, mucus, and erythrocytes. A small number of coccidia were detected in the mucosa. No lesions were seen in other parts of the alimentary tract. By immunohistochemistry, the adherent bacteria stained positive for *E coli* O15 antigen. AEEC and A/E lesions were found in the colonic tissue by transmission and scanning electron microscopy. The O15 *E coli* was not detected by culture of the rectal contents, and other tests for enteric pathogens on tissues, namely, BVD virus and *Salmonella*, were negative. *E coli* O15 is a classic EPEC serotype that causes disease naturally in rabbits (eg, strain RDEC-1) and experimentally in pigs.[8]

Pearson and colleagues[45] reported that a group of 40, 8- to 12-month old heifers in the United Kingdom developed diarrhea, and 6 developed dysentery within 1 month after being turned out to pasture in May. One 8-month-old heifer became severely ill within 5 days after the onset of diarrhea, and did not respond to antibiotic therapy. This animal collapsed, passing liquid and bloody feces, with pale mucous membranes and a hematocrit of 9.6%. The rest of the heifers responded to potentiated sulfon-amide and oral fluid therapy, and recovered. The heifer that collapsed was euthanized and immediately necropsied. A blood clot was present in the lumen of the distal

portion of the small intestine, which extended anteriorly for approximately 2 meters from the ileo-ceco-colic junction. The cecum and colon had liquid contents with small blood clots. No ulcers or sites of origin of hemorrhage in the gastrointestinal tract were visible at necropsy. *E coli* O26:K60, a known pathogenic serotype, was isolated from a sample of feces of one of the heifers, although not from the animal that was necropsied. By polymerase chain reaction (PCR), the isolate was positive for stx_1, stx_2, β-intimin, enterohemolysin, and other EHEC virulence factors. Histologically, the necropsied heifer had neutrophilic enteritis with atrophy and fusion of villi, and presence of hemosiderin-laden macrophages in the mucosa of the duodenum and ileum. The colonic mucosa contained an inflammatory infiltrate; the mucosal epithelium was irregular but intact, and colonized with bacteria on the surface. The attached bacteria stained positive for O26 antigen by immunohistochemistry. Tissue from this area excised from paraffin blocks was found to have AEEC and A/E lesions by transmission electron microscopy.

Disease in Calves Caused by E coli O157:H7 and O157:NM Infection

Diarrhea and enteritis have been associated with naturally occurring *E coli* O157:H7 infections in 1- to 3-week-old calves in Argentina, England, and South Korea.[46–48] In 1977 Ørskov and colleagues[46] isolated 3 *E coli* O157:H7 strains from the feces of a calf with enteric colibacillosis in Argentina. The exact age of this animal apparently was not determined, but was stated to be between 1 and 3 weeks. All 3 isolates were later shown to produce Stx. Ørskov and colleagues stated that although this study included only 1 diseased animal, the predominance of this serotype in the calf with diarrhea may suggest an association with disease. Further, they noted that the isolation of *E coli* O157:H7 from this calf was support for the hypothesis that cattle are a reservoir of the organism for humans. Numerous other studies later demonstrated this to be the case, as reported in many different primary research articles and summarized in review articles.[4,5,10–19]

A second report of natural association with *E coli* O157:H7 and disease in a neonatal calf was that by Daniel and colleagues[47] in the United Kingdom. These investigators isolated *E coli* O157:H7 "in pure profuse growth" from the intestine of a 6-day-old suckling calf that had developed dysentery while at pasture and died. The calf had been treated with potentiated sulfonamide boluses and oral fluid therapy. At necropsy, the calf had necrotizing enteritis, hyperemia of the mucosa of the cecum and colon, and blood-tinged fluid and cellular debris in the intestines. The calf also had a thickened umbilicus, hemorrhage from the mesenteric blood vessels along their junction with the small intestine, and enlarged mesenteric lymph nodes. The investigators noted the "calf had received an adequate intake of colostrum." Unfortunately, the intestines were not examined histologically due to advanced postmortem autolysis. The *E coli* O157:H7 isolate from the necropsied calf produced Stx2. One month later, on a return visit to the farm, rectal swabs were taken from 47 cows and 47 calves, and subsequently, Stx2+ *E coli* O157:H7 was isolated from 7 of these calves. Three of the 7 calves were 2 months old and shared a pen. The other 4 calves were 5 to 7 months old, and 2 of these shared a pen. Further testing revealed that the original isolate and 4 others were of the Stx2c subtype, and had identical pulsed field gel electrophoresis profiles.

Dean-Nystrom and colleagues[49–51] demonstrated by experimental inoculation that *E coli* O157:H7 can cause A/E lesions, enterocolitis and diarrhea in both colostrum-deprived and colostrum-fed neonatal calves, and A/E lesions without diarrhea in 3- to 4-month-old weaned calves. In addition, they demonstrated that intimin expression by the infecting organism is necessary for the formation of A/E lesions and subsequent

colonization.[50] Dean-Nystrom and colleagues[49] reported, "EHEC O157:H7 pathogenicity in cattle appears to be age related, even within the neonatal period." The virulence of EHEC O157:H7 bacteria was greater in calves inoculated when less than 12 hours old than in those 30 to 36 hours old. All calves inoculated when less than 12 hours old were colostrum-deprived, whereas calves inoculated when 30 to 36 hours old were either colostrum-fed or colostrum-deprived. Both calves less than 12 hours old and those 30 to 36 hours old at the time of inoculation developed diarrhea and A/E lesions in the small and large intestines by 18 hours PI; however, the virulence was greater in the younger calves as evidenced by the greater extent of A/E lesions in these animals. Some calves inoculated when less than 12 hours old were allowed to survive until 3 days PI, and by this time the severity of diarrhea and inflammation, and the frequency and extent of A/E lesions had increased compared with those euthanized at 18 hours PI. The colostrum was shown to contain antibodies against O157 lipopolysaccharide and Stx1, but not Stx2. Both colostrum-fed and colostrum-deprived calves inoculated with strain 3081, which is gene probe positive for stx_1 and stx_2 (as well as the intimin gene eae and CVD419, the EHEC plasmid), developed A/E lesions. Two of 3 colostrum-deprived calves compared with 1 of 4 colostrum-fed calves developed diarrhea. Calves with the most severe lesions had necrotizing, fibrinosuppurative enterocolitis associated with A/E lesions and enterocyte sloughing. Lesions were severe enough in one calf to cause severe dehydration and death by day 2 PI; this calf had been inoculated when less than 12 hours old. Collectively, A/E lesions in different calves were found in the jejunum, ileum, colon, and rectum.

Dean-Nystrom and colleagues[51] inoculated 3- to 4-month-old weaned calves with E $coli$ O157:H7 and found that the rectum was the major site of colonization. A/E lesions were seen in the rectum and cecum of calves with the highest levels of E $coli$ O157:H7. These investigators hypothesized that E $coli$ O157:H7 causes A/E lesions in older calves like those in neonatal calves, but these were not detected in earlier studies because intestinal levels of this organism at necropsy were too low (ie, $<10^6$ colony-forming units [CFU]/g of tissue) for focally distributed microscopic lesions to be detected. Because fasted ruminants were known to shed higher numbers of E $coli$ and other enteric pathogens than well-fed animals,[52] Dean-Nystrom and colleagues[51] fasted 4-month-old weaned calves for 48 hours before inoculation with EHEC O157:H7 strain 86-24 to increase intestinal levels of EHEC O157:H7 at necropsy. Fasted calves were inoculated via stomach tube with 10^{10} CFU of E $coli$ O157:H7 (9 calves) or nonpathogenic E $coli$ strain (3 calves), necropsied at 4 days PI, and examined histologically. Nine of 9 calves inoculated with E $coli$ O157:H7 and 2 of 3 calves inoculated with nonpathogenic E $coli$ developed watery diarrhea by 18 hours and 3 days after inoculation, respectively. However, 5 calves infected with E $coli$ O157:H7 and both of the diarrheic control calves had coccidia (based on histology). The occurrence of diarrhea in calves inoculated with E $coli$ O157:H7 2 days earlier than in control calves may have been evidence that this organism contributes to diarrhea in some weaned calves. At 4 days PI, higher numbers of inoculated bacteria were recovered from the intestines of weaned calves inoculated with E $coli$ O157:H7 than from the control calves. Multifocal A/E lesions were found in the rectum of 3 calves inoculated with E $coli$ O157:H7 and also in the cecum of 2 of these calves. These lesions were similar to those in neonatal calves; however, the extent of intestinal damage in the rectum and cecum in weaned calves infected with E $coli$ O157:H7 was less than that in similarly infected neonatal calves. No A/E bacteria were found in the spiral colon or ileum of any of the E $coli$ O157:H7 infected calves or in any site in control calves. The A/E bacteria were identified as E $coli$ O157:H7 by immunoperoxidase staining with anti-E $coli$ O157:H7 serum. The calves that had A/E bacteria had higher numbers of

E coli O157:H7 bacteria in the rectum and cecum than did calves in which no lesions were found. A/E bacteria were found in the 3 calves that had greater than 10^6 CFU of *E coli* O157:H7 per gram of rectal tissue, but not in those with lower counts. Two of the 3 calves with rectal lesions that had greater than 10^5 CFU of *E coli* O157:H7 per gram of cecal tissue also had A/E bacteria in the cecum. Coccidia were seen in the intestinal mucosa of 2 of the 3 calves that had A/E bacteria. These studies clearly demonstrated that weaned calves, like neonatal calves, are colonized by *E coli* O157:H7 (ie, have higher intestinal levels of inoculated bacteria at 4 days PI than do calves inoculated with a nonpathogenic control *E coli* strain) and are susceptible to intestinal damage induced by *E coli* O157:H7. High bacterial counts and A/E lesions were found only in the rectum and cecum and only in some of the calves inoculated with *E coli* O157:H7. Dean-Nystrom and colleagues[50] hypothesized that the rectum and cecum may be the principal sites of *E coli* O157:H7 colonization during the carrier-shedder state in cattle.

Escherichia coli O157:H7 Infection as a Cause of Subclinical Disease in Cattle

Naylor and colleagues[53] identified lymphoid-follicle dense mucosa at the terminal rectum as the principal site of colonization of EHEC O157:H7 in cattle, and later demonstrated the presence of A/E lesions at this location and the requirement for bacterial gene products that mediate these effects at that location.[54] In the first study,[53] colonization of the terminal rectum was detected in a 12-month-old steer that had been naturally infected, and also in experimentally infected 8- to 14-week-old calves. In the second study,[54] A/E lesions were demonstrated in 3- and 5-month-old experimentally infected calves, and the same 12-month-old naturally infected steer as the one reported previously.[53] The identification of the terminal rectum as the principal site for EHEC O157:H7 bacterial colonization and A/E lesion formation may explain why, with few exceptions,[51,55–57] these lesions were not detected in intestinal tissue in studies involving 5-day-old gnotobiotic[58] or conventional neonates and young (2–8-week-old) calves,[59–61] or cattle older than this infected with EHEC O157:H7.[60] In only one other study[51] had rectal tissue from an older (in this case 3–4-month-old) inoculated animal been examined, and in only one study had tissue from the rectum from adult cattle been shown to be susceptible to *E coli* O157:H7-induced A/E lesions; and this was in explants, not live animals.[55] Hence, the studies by Naylor and colleagues[53,54] were the first to show A/E lesions in a live, naturally infected adult (12-month-old) bovine, and first to show they were limited to the terminal rectum.

Although the terminal rectum was shown to be the principal site of *E coli* O157:H7 in cattle, in the same study Naylor and colleagues[53] found that the rumen, small intestine and proximal colon, and cecum, the last immediately distal to the ileocecal valve, are minor sites of colonization. In 2 of 54 animals studied, colonization was detected in these other sites. These findings are consistent with the results of previous experimental inoculation studies.[60,61] Other studies have demonstrated that small intestinal (ileal) tissue of conventional calves older than neonates is susceptible to A/E lesions with *E coli* O157:H7 when inoculated into ligated loops. The age of the calves in most of these studies ranged from 28 to 38 days[57,62,63]; however, as noted previously, ligated ileal loops of conventional neonatal (2- to 6-day-old) calves[44] also support these lesions. Ileal explants from an adult cow similarly were susceptible to A/E lesions.[56]

Because the ligated loop and explant models involved inoculation of a small area of tissue with a relatively great inoculum level, the development of A/E lesions in these tissues, which sometimes are not considered a major colonization site in vivo (eg,

small intestine), suggest bacterial dosage may potentially be a significant factor affecting whether A/E lesions develop in the intact host. Vlisidou and colleagues[57] reported an interesting observation in that the neuroendocrine hormone norepinephrine augmented *E coli* O157:H7-induced enteritis and adherence in the bovine calf ligated ileal loop model, and these effects were dependent on the ability of *E coli* O157:H7 to induce A/E lesions. Epinephrine and norepinephrine cross-talk with a bacterial quorum-sensing system regulating expression of genes that encode for numerous protein virulence factors involved in colonization, for example, the type III secreted proteins and flagellum.[64] Quorum-sensing is a mechanism that the bacteria use to sense the density of their species in an environment and colonize an area like the mucosal surface. Quorum-sensing is an intriguing area of research currently under investigation with regard to mechanisms by which EHEC O157:H7 and other AEEC colonize and cause disease.

Other studies have demonstrated that inflammation, innate immune responses, and systemic and mucosal antibody responses occur in response to *E coli* O157:H7 colonization and infection of the intestinal mucosa in cattle of various ages, including adults, and these findings are now taken as evidence that this organism is a pathogen of the bovine host. Bretschneider and colleagues[65–67] demonstrated that adult cattle (mean age, 16 months) orally inoculated with *E coli* O157:H7 and shedding this organism in their feces developed antigen-specific IgA and IgG rectal mucosal and IgG serum antibodies to O157 lipopolysaccharide, and to type III secreted proteins known to be involved in A/E lesion formation, namely EspA, EspB, and the translocated intimin receptor (Tir). Collectively, Dean-Nystrom and colleagues,[50] Cornick and colleagues,[68] Naylor and colleagues,[54] and Vlisidou and colleagues,[63] through the use of bacterial constructs containing deletions in genes encoding intimin (*eae*) and type III secreted proteins, namely Tir (*tir*), demonstrated that the A/E attachment mechanism mediated by these gene products plays a critical role in colonization of the bovine intestine, including the terminal rectum. Bretschneider and colleagues[66] reported that *E coli* O157:H7 strains that expressed reduced amounts of H7 flagellin protein including those that were completely nonflagellated did not effectively colonize the intestines of inoculated adult cattle. The results of a study by Dobbin and colleagues[69] suggested that a regulator of flagellar gene expression, and not the flagella itself, played a significant role in colonization. However, Mahajan and colleagues[70] demonstrated that H7 flagella facilitate attachment of *E coli* O157:H7 to bovine rectal epithelial cells in vitro. These investigators hypothesized that the H7 flagellum does indeed play a significant role in colonization, first through its role in providing motility, and second by "browsing" the epithelial surface, then tethering the bacterium to accessible receptors, for example, mucin or other glycoconjugates. Colonization is then thought to progress into a secondary phase involving a decrease in expression of the flagella, and an increase in expression of type III secreted proteins with A/E lesion formation. Naylor and colleagues[71] and Nart and colleagues[72] demonstrated that cattle develop humoral and mucosal antibody responses, respectively, against O157, H7, and other antigens in response to colonization. Antibodies of the IgG and IgA class were both found in the serum and rectal mucosa. Bretschneider and colleagues,[65] and Naylor and colleagues[71] both found that circulating antibodies of the IgA class decrease in titer following repeated bacterial challenge, suggesting that the circulating antibodies are consumed, perhaps by translocation into the gut lumen, in response to infection. Bretschneider and colleagues[65] found that IgA antibodies to type III secreted proteins and not those to O157 lipopolysaccharide (LPS) decreased after the second challenge. Both studies also demonstrated that cattle shed less bacteria in the feces on subsequent challenges. Hence, antibodies directed

against proteins that mediate colonization are used for protection against subsequent challenge. Nart and colleagues[73] reported that *E coli* O157:H7 infection of cattle induced a quantifiable neutrophilic inflammatory response in the lamina propria of the rectum when bacterial numbers reached 10^5 CFU/cm^2. These animals also developed IgA antibody responses to whole *E coli* O157:H7 cells. The investigators concluded that, based on the identification of a pathologic change and the production of a local immune response in the terminal rectum, *E coli* O157:H7 should not be considered a commensal organism in cattle.[73]

Proposed Role of Shiga Toxin in the Bovine Host

Mainil and Daube[12] reported in a major review article that more than 85% of EHEC isolated from cattle are positive for stx_1 only (as opposed to stx_2, both stx_1 and stx_2, or no *stx*), and this group of AEEC in particular are diarrheagenic pathogens of calves. An important question is the role of Stx, if any, in EHEC colonization of the bovine intestine. Stx is a potent cytotoxin that plays a key role in the induction of vascular lesions and other pathogenetic events that characterize HC and HUS in humans. However, unlike human beings, cattle lack the receptor for Stx in their blood vessels, and this has been proposed as a major reason why cattle do not usually develop severe hemorrhagic colitis and have never been found to develop HUS following EHEC infection.[74] Cattle do, however, have receptors for Stx in some cells, and this may potentially contribute to disease. Hoey and colleagues[75] reported the finding of Stx receptors in crypt epithelial cells in the intestine, but the toxin does not traffic to the endoplasmic reticulum; it ends up in lysosomes, which probably prevents toxicity. Stamm and colleagues[76] identified novel mesenchymal, nonepithelial Stx target cells in the crypt area of the bovine colonic mucosa. These investigators reported that bovine crypt epithelial cells are resistant to the effects of Stx1, but some intestinal mucosal mesenchymal cells, preliminarily characterized as mucosal macrophages, are Stx1-responsive and may participate in the interaction of STEC with the bovine intestinal mucosa. Menge and colleagues[77] found that intraepithelial lymphocytes in cattle have receptors for Stx, and reported that Stx1 reduces the proliferative responses of these cells to mitogenic stimuli, and reduces expression of CXCL8 (interleukin-8) by these cells. Hoffman and colleagues[78] found that the development of a cellular immune response against STEC antigens is significantly delayed in calves following inoculation with Stx2-producing *E coli* O157:H7. Fecal shedding of Stx2+ O157 was significantly higher than that of Stx-nonproducing *E coli* O157:H7. This shedding occurred despite the development of antibodies against O157 LPS. Hoffman and colleagues[78] hypothesized that Stxs cause immunosuppression by interfering with antigen-specific cell-mediated immune responses, which promote STEC colonization in the bovine host.

EVIDENCE OF AEEC AS CATTLE PATHOGENS BASED ON EPIDEMIOLOGIC STUDIES
Escherichia coli O5, O26, O111, O118, and O145 Infection of Calves

Several serogroups of AEEC and STEC have been isolated from diarrheic calves in different countries. In the United Kingdom, Sherwood and colleagues[79] reported that 13 of 306 (3%) of diarrheic calves had *E coli* in their feces that produced detectable Stx, and they belonged to the O4, O8, O19, O26, O111, O149, O168, and O nontypeable groups. Serogroup O111 was isolated from 3 calves, O26 was isolated from 2 calves, and the others were isolates from one each. Two calves were concurrently infected with *Cryptosporidium* spp, and one with rotavirus and coronavirus. The isolates were not tested for the intimin gene; hence, it was not reported whether these

were AEEC. Wieler and colleagues[80] reported that out of 174 *E coli* strains isolated from diarrheic calves in Germany and Belgium, that were positive for *stx* genes, 122 strains (70.1%) were also positive for *eae*. One hundred 7 of these *eae*-positive strains (87.7%) harbored stx_1 genes, 13 strains (10.7%) had stx_2 genes, and 2 strains (1.6%) had both *stx* genes. The strains displayed 17 different O types, the majority (97 strains [79.5%]) belonging to O5 (5 strains), O26 (21 strains), O111 (13 strains) O118 (36 strains), O145 (9 strains), and O157 (13 strains).

Orden and colleagues[81] screened fecal samples from 221, 1- to 30-day-old diarrheic dairy calves in Spain. A total of 861 culture isolates from these samples were identified as *E coli*. *E coli* isolates were tested for Stx first by Vero cell assay and then for *stx* genes by PCR; if an isolate was positive by either method it was called STEC. All isolates that were positive for Stx by Vero cell assay were found to be *stx*+ by PCR. Isolates also were tested by PCR for *eae* and *espB*. STEC (in this study this term included all Stx+ that were either *eae*+ or *eae*−) and *eae*+ non-STEC were detected in 20 (9.0%) and 18 (8.1%) of the diarrheic calves tested, respectively. Of the STEC, 69.8% were positive for stx_1, 20.9% were positive for stx_2, and 9.3% were positive for stx_1 and stx_2. STEC isolates in a high percentage (76.7%) of diarrheic dairy calves belonged to the O4, O26, O39, O91, O113, O128, and O145 serogroups. Data from calves was grouped according to age as follows: 1 to 7 days, 8 to 14 days, 15 to 21 days, and 22 to 30 days; and odds ratios (OR) with 95% confidence intervals for infection with STEC and *eae*-positive non-STEC were calculated. The odds of STEC infection in 22- to 30-day-old diarrheic calves was significantly higher in comparison with the 1- to 7-day-old (OR = 14.09), 8- to 14-day-old (OR = 8.18), and 15- to 21 day-old (OR = 5.73) calves. The odds of infection with *eae*-positive non-STEC in the 22- to 30-day-old calves was greater in comparison with the 1- to 7-day-old calves (OR = 5.74).

China and colleagues[82] conducted a large-scale epidemiologic study in Belgium that addressed whether AEEC are pathogens of calves. A total of 695 calves were included in the study: 295, 2- to 10-week-old calves that had died of diarrheal disease; 311, 4- to 6-week-old healthy calves from 5 different farms without a serious diarrheal problem; and 89 newborn to 3-month-old calves from 7 farms with severe diarrhea problems. In the case of animals that died, intestinal contents instead of feces was cultured, and they were sampled only once (ie, post mortem). In the case of healthy calves on farms without a diarrhea problem, fecal samples were collected only once. Fecal samples were collected twice per week for 12 weeks from calves with or without diarrhea on farms that had a history of recurrent diarrheal disease. Farms with a history of recurrent diarrhea had these signs despite vaccination for ETEC, rotavirus, coronavirus, and antibiotic therapy. Both colony hybridization with gene probes and PCR were used to assay for *eaeA*, *espB*, stx_1, and stx_2. Strains were called AEEC if they had a positive result for *eaeA*; EPEC if they were *eaeA*+ but negative for *stx*; VTEC (STEC) if they were *stx*+, but negative for *eaeA*; and EHEC if they were *eaeA*+ and *stx*+. AEEC strains were identified in 91% of calves on farms with recurrent diarrhea problems, and in 66% of the calves, there was a correlation between the presence of AEEC and diarrhea. Feces from 1- to 12-week-old calves were positive for AEEC; 90% of these samples came from 2- to 8-week-old calves, and the mean age of the calves with AEEC was 5 weeks. The number of Stx positive bacteria was significantly higher in calves that died of diarrhea than in healthy or sick calves, and this finding was interpreted as evidence that Stx played an underlying role in the pathogenesis of disease. China and colleagues[82] stated that AEEC are clearly present in large numbers in farms with recurrent problems of diarrhea with variation from farm to farm. The proportion of calves with diarrhea and AEEC (66%) was significantly higher than

the proportion of healthy calves with AEEC (25% in farms with diarrhea and 24% in farms without diarrhea).

Lee and colleagues[83] investigated the rates of occurrence of EHEC O26 and O111 in calves with or without diarrhea on farms in South Korea. These investigators conducted an observational study involving a total of 442 diarrheic and nondiarrheic young beef and dairy calves (<16 weeks old) from 115 different farms. EHEC O26 and O111 were detected in 14.4% and 12.5% of the diarrheic calves, respectively, compared with 7.6% and 5.9% of the nondiarrheic calves. The authors of this article (R.A.M., D.R.S.) analyzed the data from this study using multivariate logistic regression to evaluate the effect of calf age and clinical signs of diarrhea on the probability to recover EHEC O26 and O111 from these calves. There were significant (*P*<.05) interactions between the age of the calf and presence of diarrhea on the probability for recovering EHEC O26 or O111 from the feces. Of 163 calves 3 weeks old or less, 18 of 110 (16%) calves with diarrhea, and 1 of 53 (2%) calves without diarrhea shed EHEC O26. Of the 279 older calves, 19 of 147 (13%) calves with diarrhea shed EHEC O26, and 13 of 132 (10%) calves without diarrhea shed EHEC O26. Among calves 3 weeks old or less, the odds for calves with diarrhea to be shedding EHEC O26 was 10.2 times greater than the odds for nondiarrheic calves; yet, among calves older than 3 weeks the odds for calves with diarrhea to be shedding EHEC O26 was only 1.4 times greater. Of 239 calves 4 weeks old or less, 21 of 157 (13%) calves with diarrhea, and 2 of 82 (2%) calves without diarrhea shed EHEC O111. Of the 203 older calves, 11 of 100 (11%) calves with diarrhea shed EHEC O111, and 9 of 103 (9%) calves without diarrhea shed EHEC O111. Among calves 4 weeks old or less, the odds for calves with diarrhea to shed EHEC O111 was 6.2 times greater than the odds for nondiarrheic calves; yet, among calves older than 4 weeks the odds for calves with diarrhea to shed EHEC O111 was only 1.3 times greater. These interactions between age and diarrhea on recovery of EHEC O26 and O111 suggest that neonatal calves were more likely to exhibit clinical signs with these infections than older calves.

Pearce and colleagues[84] conducted a study in northern Scotland that investigated the shedding of *E coli* O26, O103, O111, O145, and O157 in a cohort of beef calves from birth over a 5-month period. *E coli* O26 was shed by 94% of the calves, and more than 90% of the O26 isolates were positive for stx_1, *eae*, and enterohemolysin (*ehl*) genes. *E coli* O103 was shed by 51% of the calves. Forty-eight percent of the O103 isolates were positive for *eae* and *ehl*; none were positive for stx_1. No O111 was detected, and shedding of O145 and O157 was rare. All but one O157 isolate was positive for stx_2, *eae*, and *ehl*. No association between fecal shedding of *E coli* O26 and O103 and diarrhea in the calves was found. A major finding was that the pattern of shedding of O26 and O103 in calves was very different. *E coli* O26 was shed by a higher proportion of dams at calving than at the end of the study; this suggested higher O26 exposure to newborn calves from the dam; in addition the stx_1, *eae*, and *ehl* genes were more common in O26 than O103 and the investigators hypothesized that the products of these genes might play an important role in colonization in the calf gut. With regard to why no relationship between shedding and diarrhea was detected, Pearce and colleagues suggested that the dams may have transferred protective antibodies in the colostrum to their calves, thereby preventing diarrhea; however, this was not tested. The investigators also commented that because calves were sampled weekly, they may have missed diarrheic episodes lasting less than 7 days.

Blanco and colleagues[22] characterized the virulence factors and O groups of 514 STEC isolates from diarrheic and healthy cattle in Spain. The isolates belonged to

164 different seropathotypes (associations between serotypes and virulence genes); however, only 12 accounted for 43% of the isolates. Seropathotype O157:H7 stx_2 eae ehxA (46 isolates) was the most common, followed by O157:H7 stx_1 stx_2 ehxA (34 isolates), O113:H21 stx_2 (25 isolates), O22:H8 stx_1 stx_2 ehxA (15 isolates), O26:H11 stx_1 eae ehxA (14 isolates), and O77:H41 stx_2 ehxA (14 isolates). STEC isolates belonged to 66 O serogroups and 113 O:H serotypes, including 23 new sero-types. Sixty-seven percent belonged to 1 of 15 serogroups, namely, O2, O4, O8, O20, O22, O26, O77, O91, O105, O113, O116, O157, O171, O174, and OX177. Fifty-two percent belonged to only 10 serotypes, namely, O4:H4, O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, O171:H2, and ONT:H19. The eae (intimin) genes were subtyped and a new variant, namely, eae-ξ, was discovered. With this discovery, at least 15 different intimin types and subtypes have now been identified (α1, α2, β1, β2, γ1, γ2/θ, δ/κ, ε, ζ, η, ι, λ, μ, ν, and ξ). The extensive number of combinations serotypes, coupled with variations of virulence factors (seropatho-types) highlights the increasingly complex diversity of STEC infections in cattle. In this study, the overall prevalence rates of STEC colonization were estimated to be 37% in calves and 27% in cows. Isolates that were positive for stx_2 and those positive for both stx_1 and stx_2 were present in similar proportions in calves and cows. In contrast, isolates that were positive for eae and stx_1 were more commonly recovered from calves than cows. Although they did not report an analysis of data comparing the diarrheic versus healthy calves in this study, these investigators cited their previous surveys in which they found a significantly higher percentage of Stx1-producing E coli in diarrheic calves, which suggested a pathogenic role in neonatal calf diarrhea.

Aidar-Ugrinovich and colleagues[85] conducted a study that investigated the occur-rence, serotypes, and virulence markers of STEC and EPEC strains in diarrheic and nondiarrheic calves in Brazil. A total of 546 fecal samples from 264 diarrheic calves and 282 healthy calves on beef farms in São Paulo were screened by PCR. STEC and EPEC were isolated in 10% and 2.7% of the 546 animals, respectively. E coli O157:H7 was not detected in any of the calves. The most frequent serotypes among STEC strains detected were O7:H10, O22:H16, O111:H⁻, O119:H⁻, and O174:H21; the most prevalent EPEC strains detected were O26:H11, O123:H11, and O177:H11. Several serotypes detected in this study constituted ones not previously reported among STEC, namely, O7:H7, O7:H10, O48:H7, O111:H19, O123:H2, O132:H51, O173:H⁻, and O175:H49. In this study, the investigators found no differ-ences in carriage of STEC and EPEC in diarrheic and healthy cattle; however, they stated that "a significantly higher number of Stx1-producing E coli were found in diar-rheic calves, suggesting a pathogenic role in neonatal calf diarrhea."

Escherichia coli O157:H7 and O157:NM Infection of Calves

Kang and colleagues[48] conducted an observational study similar to a previous one by this group involving EHEC O26 and EHEC O111[83] to investigate the rates of occur-rence of EHEC O157:H7/NM in diarrheic and nondiarrheic calves younger than 20 weeks on farms in South Korea. A total of 498 diarrheic and nondiarrheic calves on 115 farms were included. EHEC O157:H7/NM was detected in 24 of 244 (10%) of the diarrheic calves, respectively, compared with 7 of 254 (3%) of the nondiarrheic calves. The authors of this article (R.A.M., D.R.S.) analyzed the data from this study using multivariate logistic regression to evaluate the effect of calf age and clinical signs of diarrhea on the probability to recover EHEC O157:H7/NM from these calves. Anal-ysis revealed a significant ($P = .001$) interaction between the age of the calf and pres-ence of diarrhea on the probability for recovering EHEC O157:H7/NM from the feces. Of 194 calves 4 weeks of age or less, 17 of 125 (14%) calves with diarrhea, and 1 of 69

(1.4%) calves without diarrhea shed EHEC O157:H7/NM. Of the 304 older calves, 7 of 119 (6%) calves with diarrhea shed EHEC O157:H7/NM, and 6 of 185 (3%) calves without diarrhea shed EHEC O157:H7/NM. Among calves 4 weeks old or less, the odds for calves with diarrhea to be shedding EHEC O157:H7/NM was 10.7 times greater than the odds for nondiarrheic calves; yet, among calves older than 4 weeks the odds for calves with diarrhea to be shedding EHEC O157:H7/NM was only 1.9 times greater. The interaction between age and diarrhea on recovery of EHEC O157:H7/NM was nearly identical to those observed with EHEC O26 and O111, and suggests that neonatal calves were more likely to exhibit clinical signs with EHEC O157:H7/NM infection than older calves.

SUMMARY

A review of the literature provides overwhelming evidence that AEEC are pathogens of cattle, mainly calves between the ages of 1 and 5 weeks. AEEC predominantly infect the large intestine and may cause diarrhea, with roughly one-fourth of the cases manifested clinically as dysentery, and pathologically as colitis or enterocolitis. AEEC infections are usually seen in conjunction with other enteric pathogens, for example, *Cryptosporidium* spp, other coccidia, rotavirus, and coronavirus, and these organisms significantly increase the severity of the clinical illness. In neonatal calves and in some cases, older animals, the infections can be fatal, but mainly when superimposed on another enteric infection. Most cases of clinical illness are caused by serogroups O5, O26, O111, O118, and O145, but other serogroups may be involved, which varies in different parts of the world. *E coli* O157:H7 and O157:NM are pathogens of the neonate, but epidemiologic evidence suggests they may be associated with diarrheal disease in older calves. By definition, AEEC cause attaching-effacing lesions, which are ultrastructural, and characterized by effacement of microvilli and intimate attachment of the bacterium to the apical cell membrane of the enterocyte. The causation of A/E lesions requires products of bacterial genes that induce microvillous effacement and intimate attachment. These genes are located on the locus of enterocyte effacement on the bacterial chromosome and their products include, but are not limited to, type III secreted proteins and the outer membrane protein, intimin. Most clinically apparent AEEC infections in calves are caused by strains that, by definition, produce intimin but also typically produce Shiga toxin (Stx), and especially Stx1. Although cattle do not have vascular receptors for Stx, they do have receptors on intraepithelial lymphocytes and cells that have preliminarily been identified as mucosal macrophages in the intestine. By acting on these cells, Stx is thought to cause immunosuppressive effects, especially affecting cell-mediated immune responses, and these may interfere with the ability of the host to clear the infection or develop fully protective immunity on recovery. AEEC pathologically are most likely to cause clinically evident disease when bacterial colonization is extensive enough to cause significant sloughing of enterocytes. Enterocyte loss and inflammation may be severe enough to result in diarrhea and dysentery.

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