INFECTION AND IMMUNITY, Oct. 1994, p. 4325–4332
0019-9567/94/$04.00+0

Vol. 62, No. 10

Development of Large Intestinal Attaching and Effacing Lesions in Pigs in Association with the Feeding of a Particular Diet

N. A. NEEF,[1]* S. McORIST,[2] R. J. LYSONS,[1] A. P. BLAND,[1] AND B. G. MILLER[3]

AFRC Institute for Animal Health, Compton, Newbury, Berkshire RG16 0NN,[1] Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Veterinary Field Station, Easter Bush, Midlothian EH25 9RG,[2] and School of Veterinary Science, University of Bristol, Langford, Bristol BS18 7DU,[3] United Kingdom

Received 2 May 1994/Returned for modification 24 June 1994/Accepted 6 July 1994

Hysterotomy-derived piglets were kept in gnotobiotic isolators and artificially colonized at 7 days of age with an adult bovine enteric microflora. At 3 weeks of age, the pigs were transferred to conventional experimental accommodation and weaned, either onto a solid diet that had been associated with field cases of typhlocolitis in pigs or onto a solid control diet. At necropsy at 5 weeks of age, groups of pigs fed the diet associated with field cases of typhlocolitis were found to have developed typhlocolitis. This was absent from the groups fed the control diet. The typhlocolitis was characterized by attaching and effacing lesions typical of those described following experimental inoculation of various species with enteropathogenic *Escherichia coli*. A nonverocytotoxic, *eae* probe-positive *E. coli* serotype O116 was isolated from pigs on the colitis-associated diet but not from any of the pigs on the control diet. Coliform bacteria attached to the colonic lesions reacted with polyclonal antiserum to *E. coli* O116 in an immunoperoxidase assay of histological sections of affected tissue. No reaction with this antiserum was observed in corresponding tissue sections taken from pigs on the control diet. No colon lesions were observed in germfree pigs fed either of the diets. It is postulated that proliferation and possibly expression of pathogenicity of the attaching and effacing *E. coli* responsible for the lesions are strongly influenced by diet.

Bacterial attachment to intestinal epithelial cells with effacement of the surface-absorptive microvilli is a property of certain strains of *Escherichia coli* and has also been described in mice in association with strains of *Citrobacter freundii* (21). In the case of *E. coli*, the so-called attaching and effacing strains, AEEC (27), include both enteropathogenic *E. coli* strains (EPEC) and enterohemorrhagic *E. coli* strains. EPEC are a cause of diarrhea in human infants, rabbits (6), calves (14), and possibly other species (18). In contrast to most enterohemorrhagic *E. coli* strains, which produce at least one of two cytotoxins, factors contributing to the clinical effects of EPEC on the intestines remain relatively obscure (7, 11). Both gnotobiotic and neonatal conventional pigs have been used successfully as experimental models for demonstrating the attaching and effacing properties of *E. coli* strains associated with diarrhea in humans and other species (13, 27, 39). In these models, the principal site of lesions caused by these organisms is always the large intestine. Experimental reproduction of attaching and effacing lesions in neonatal pigs, using an *E. coli* strain of pig origin, has been described (16), but weaned pigs with a more adult-type fermentative large intestinal flora have not been used. Weaning onto solid food has been suggested as a predisposing factor for the production of diarrhea secondary to attaching and effacing lesions in rabbits (9, 31) but not as a factor predisposing to the lesions themselves. The composition of the diet itself has not been suggested as a predisposing factor for the production of this type of lesion in any species.

These experiments were conducted in order to investigate a reported association of particular feeds with the development of nonspecific diarrhea and colon lesions in weaned pigs, known as porcine colitis (12, 33, 36, 40). The effects on the large intestine of a typical dietary formulation associated with this condition were compared with those of a control diet in both germfree (GF) and artificially "conventionalized" (CV) piglets, with a predominantly anaerobic large intestinal flora.

MATERIALS AND METHODS

Experimental design. Six GF piglets (Large White × Landrace) were delivered by hysterotomy and maintained in plastic isolators as described previously (35). GF status was confirmed with weekly rectal swabs, cultured both aerobically and anaerobically. GF pig experiments were carried out on two separate occasions, and all data were pooled. At 14 days of age, the pigs were weaned from a canned evaporated cows' milk diet onto a control diet, diet 1 (described below). At 21 days of age, four pigs were transferred to diet 2 and two remained on diet 1. Feeds were given ad libitum, with sterile plain water always available. All pigs were killed for postmortem examination at 35 days of age.

CV pig experiments were carried out on two separate occasions (experiments A and B), using 16 pigs (Large White × Landrace) in experiment A and 10 pigs (Large White × Landrace × Duroc hybrids) in experiment B. The piglets were derived by hysterotomy, dipped in povidone iodine solution (Pevidine; BK Veterinary Products, Bury St. Edmunds, United Kingdom [U.K.]), and placed immediately into sterile plastic isolators in groups of between 10 and 17 pigs. The groups were fed and maintained in the same manner as the gnotobiotic pigs. At 7 days of age, all pigs were orally inoculated with an enteric flora (described below). The pigs were removed from the isolators at 14 days of age and transferred to rooms maintained at approximately 28°C with no bedding. All rooms had been previously fumigated with formalin gas, and staff caring for the pigs wore plastic suits and gloves which were disinfected after each use. Air supply entering and leaving the rooms was

* Corresponding author. Present address: Division of Comparative Medicine, Johns Hopkins University School of Medicine, 720 Rutland Ave., Baltimore, MD 21205-2196. Phone: (410) 955-3273. Fax: (410) 550-5068.

TABLE 1. Dietary formulations

Component	Amt (%)
Diet 1	
Skimmed milk powder	60.70
Fats and oils	20.67
Whey	6.25
Delactosed whey	4.88
Pregelatinized starch	2.50
Organic acid salt	1.00
Inert flow agent	1.00
Vitamin and mineral supplement	0.50
Digestible energy	19.8 MJ/kg
Crude protein	24.00
Fat	21.00
Ash	7.50
Moisture	3.50
Diet 2	
Wheat	25.15
Soya extract (48%)	22.72
Extruded full-fat soya	15.00
Tapioca	15.00
Skimmed milk powder	10.00
Field peas	5.00
Cane molasses	2.50
Soya oil	1.50
Dicalcium phosphate	1.38
Salt	0.57
Milk supplement	0.50
L-Lysine HCl	0.26
Limestone	0.19
DL-Methionine	0.14
L-Threonine	0.09
Digestible energy	14.86 MJ/kg
Crude protein	24.00
Fat	6.00
Crude fiber	3.50
Moisture	10.30

filtered through HEPA filters. Pigs were fed on the same canned evaporated milk diet as was fed in the isolators until 21 days of age, when they were divided randomly into two groups, placed in two separate rooms. EDTA blood samples were taken from each pig, and total and differential leukocyte counts were performed. At this point, the animals were weaned abruptly onto either diet 1b or diet 2 (described below), fed ad libitum from troughs. In experiment B only, the feed consumption of each diet group was recorded. EDTA blood samples were taken from all pigs for total and differential peripheral leukocyte counts, and each pig was weighed before euthanasia for postmortem examination at 35 days of age.

Enteric flora. The experimental enteric flora was derived from the feces of a healthy adult bovine and passaged 13 times in milk-fed GF pigs and calves kept in gnotobiotic isolators (28). Between passages, feces were diluted 1:4 in prereduced brain heart infusion broth (Oxoid; Unipath Ltd., Basingstoke, U.K.) with 10% glycerol and stored at −70°C. Other than a brief elevation of rectal temperature, no clinical signs were observed in the animals used for passage of the flora. There were no gross or histopathological lesions in large and small intestinal tissue sections taken from sample animals. Freedom from pathogenic enteric viruses was therefore assumed. Neither salmonellae, *Yersinia* spp., nor spirochetes of any description could be cultured from this flora by standard techniques, including enrichment for both salmonellae and *Yersinia* spp.

TABLE 2. Histological damage, fecal dry-matter content, and body weight

Expt (no. of pigs)	Diet	Attaching and effacing score (mean per pig)[a]					Mean (SD)	
		Ileum	Cecum	PC	MC	DC	Fecal dry matter (%)	Wt (kg)
A (8)	1b	0	0	0	0	0	29 (4)	7.0 (1.0)
A (8)	2	0.75	2.00	2.13	2.00	1.38	29 (5)	6.0 (0.7)
B (5)	1b	0	0	0	0	0	26 (3)	6.8 (0.8)
B (5)	2	0.20	1.40	2.00	2.20	1.40	22 (1)[b]	4.7 (0.7)[c]
GF (2)	1	0	0	0	0	0	15	NT[d]
GF (4)	2	0	0	0	0	0	20 (2)	NT

[a] PC, proximal colon; MC, midcolon; DC, distal colon.
[b] Significant ($P < 0.05$; two-sample t test).
[c] Significant ($P < 0.005$; two-sample t test).
[d] NT, not tested.

Experimental diets. The formulations of diets 1 and 2 are given in Table 1. Diet 2 was formulated to resemble diets that had been associated with nonspecific diarrhea in weaned pigs in the field. Diet 1, the control diet, was based largely on powdered skimmed cows' milk. Both diets were pelleted and sterilized by irradiation at 5 Mrads. In the CV pig experiments, diet 1 was mixed with wheat bran (280 g of bran to 720 g of diet 1 per kg of feed [diet 1b]) to equalize the crude fiber content of the two diets at 3.5%.

Postmortem examination. All pigs were killed by electrical stunning and exsanguination. Samples of the terminal ileum (15 cm proximal to the ileo-cecal valve), cecum (blind end), proximal colon (first loop), midcolon (sigmoid flexure), and distal colon (final loop) were collected within 3 min of death and fixed in 10% formol-buffered saline. The pHs of the contents of the terminal ileum, cecum, proximal colon, and feces were determined in situ, and samples were taken for osmolality determination. The dry-matter content of fecal samples was determined by drying a known weight of feces in an oven at 80°C and weighing daily until no further weight loss occurred. Dry-matter content was calculated as a percentage of the wet weight of each sample.

Bacteriology. Fecal samples from all experimental pigs were cultured for the presence of certain bacteria as follows. Spirochetes were grown anaerobically at 39°C for up to 7 days on Fastidious Anaerobe Agar (LabM) supplemented with 400 mg of spectinomycin per ml and identified by their morphological appearance under phase-contrast microscopy. *E. coli* bacteria were cultured aerobically on sheep blood agar (Oxoid Blood Agar Base no. 2 supplemented with 5% defibrinated ovine blood) for 24 h at 37°C and identified by using the API 20E system (Biomerieux). *Yersinia* spp. were detected by aerobic incubation of CIN agar (Oxoid Yersinia Selective Agar) at 28°C for 48 h. The identity of sample colonies was confirmed with the API 20E system (Biomerieux). Salmonellae were detected by inoculation of Rappaport broth and aerobic incubation at 40°C for 48 h, followed by subculture on brilliant green agar. Nonselective culture for other bacteria included aerobic (37°C) and anaerobic (39°C) incubation of sheep blood agar for 24 and 48 h, respectively.

In experiment B only, the numbers of aerobic and anaerobic bacteria were also estimated by successive 1:10 dilutions of

TABLE 3. Physical properties of intestinal contents[a]

Expt	Diet	Ileum		Cecum		Proximal colon		Feces pH
		pH	Osmolality (mosmol/liter)	pH	Osmolality (mosmol/liter)	pH	Osmolality (mosmol/liter)	
A	1b	7.37 (0.32)	341 (18)	6.44 (0.25)	386 (34)	6.50 (0.31)	386 (25)	6.87 (0.34)
A	2	7.26 (0.23)	349 (24)	6.71 (0.18)	390 (30)	6.49 (0.18)	399 (32)	6.69 (0.24)
B	1b	7.05 (0.40)	346 (24)	6.07 (0.21)	447 (16)	5.95 (0.28)	451 (19)	6.29 (0.37)
B	2	7.07 (0.24)	367 (29)	6.08 (0.10)	417 (26)	6.14 (0.15)	429 (27)	6.60 (0.24)
GF	1	8.02 (0.04)	361 (21)	7.95	333	7.78 (0.24)	331 (1)	7.51 (0.30)
GF	2	8.36 (0.04)	330 (18)	8.15 (0.13)	344 (30)	8.12 (0.21)	332 (8)	7.25 (0.31)

[a] Values are means for each group; standard deviations are given in parentheses.

fecal samples, using prereduced phosphate-buffered saline in an anaerobic cabinet. The various dilutions were plated onto sheep blood agar and incubated aerobically and anaerobically for 48 h. Further selective culture was performed for *Clostridium difficile* (3) and *Bacteroides fragilis* (5). Sorbitol MacConkey agar (Oxoid) was also used to identify sorbitol-nonfermenting *E. coli* strains. After selective culture, colonies of interest were then identified biochemically, using API 20A (anaerobic species) or API 20E (members of the *Enterobacteriaceae* family). The predominant *E. coli* isolates from nonselective culture from each diet group, any sorbitol-nonfermenting strains, and any other strains of interest were characterized further at the Public Health Laboratory Service, London, U.K., in standard hybridization reactions with the *eae* probe for attaching and effacing ability (20), the VT1 and VT2 probes for Vero cytotoxin genes 1 and 2 (41), and in standard serogroup reactions with rabbit polyclonal antisera.

Osmolality of intestinal content. Samples were stored at 4°C and processed on the day of collection. Intestinal contents were centrifuged at 12,000 rpm in a microcentrifuge (Eppendorf centrifuge 5415C; Andermann & Co., Kingston upon Thames, U.K.), and the osmolality of the supernatant was determined by freezing-point depression, using an MSE Wide-Range Advanced Osmometer (Advanced Instruments, Inc., Needham Heights, Mass.).

Scanning electron microscopy. Formalin-fixed material from seven pigs (two from each diet group in experiment A, two from the diet 2 group, and one from the diet 1b group in experiment B) was postfixed in 1% phosphate-buffered osmium textroxide, dehydrated to acetone and critical-point dried, coated with platinum, and then examined with a Hitachi S-520 scanning electron microscope at an accelerating voltage of 20 kV.

Histological sections. Formalin-fixed tissue was embedded in paraffin wax and processed by using standard procedures. All sections were cut at 3-μm thickness and stained with hematoxylin and eosin, periodic acid-Schiff-alcian blue, and Gram stains. Further sections from both diet groups were cut, dewaxed, and used in an indirect immunoperoxidase assay for *E. coli* O116. This utilized rabbit polyclonal antisera to *E. coli* serogroup O116 and secondary antisera to rabbit immunoglobulin G conjugated to horseradish peroxidase, with AEC (aminoethylcarbazole) substrate (Vector Laboratories). Rabbit antisera to other serogroups of *E. coli* were included as negative controls in each batch of assay reactions.

Morphometrics. Villus height and crypt depth of longitudinally sectioned crypt-villus pairs were measured on the hematoxylin-eosin-stained terminal ileum sections, using a MOP3 apparatus (Kontron, Echung/Munich, Germany). A minimum of seven pairs from each pig were measured, and a mean crypt/villus ratio was calculated for each experimental group.

Estimation of large intestinal mucin content. Differences in the large intestinal mucin content of each of the diet groups were estimated by image analysis, using the periodic acid-Schiff-alcian blue-stained sections. The mean percentage of stained mucin per unit area of fully sectioned lamina propria was determined for each region of the large intestine, using an Optomax V image analyzer system (Analytical Measuring Systems Ltd., Shirehill, Essex, U.K.). All sections from a single experiment were examined in a single session. Five to 12 readings were taken from each section, using a ×4 objective, such that a minimum of 10 mm^2 of full-thickness lamina propria was analyzed in each case. Mean readings were calculated for each region of the large intestine for each experimental group.

Evaluation of attaching and effacing lesion. The degree of surface epithelium affected by degenerative lesions was estimated for each of the large intestinal hematoxylin-eosin-stained sections, and grades of 0 to 3 were awarded, using a system similar to that described by Moon et al. (27). Grade 0 indicated that no attaching and effacing lesions were seen; grade 1, less than 10% of the surface epithelium was affected; grade 2, 10 to 50% of the surface epithelium was affected; and grade 3, 50% or more of the surface epithelium was affected. The criterion used for defining an attaching and effacing lesion was the presence of either bacteria attaching to depressions in the surface of enterocytes or areas of complete epithelial erosion that were adjacent to areas where attaching and effacing bacteria could be seen. A mean score for each region

TABLE 4. Peripheral leukocyte parameters[a]

Expt (no. of pigs)	Diet	Total leukocytes (10^9) per liter	Neutrophils (10^9) per liter	Lymphocytes (10^9) per liter
A (8)	1b	7.05 (1.40)	2.54 (0.91)	4.46 (1.08)
A (8)	2	15.52 (5.07)[b]	11.28 (4.87)[b]	4.08 (1.00)
B (5)	1b	8.20 (4.20)	3.93 (5.74)	4.12 (1.13)
B (5)	2	22.90 (8.80)[c]	12.87 (5.74)[c]	9.92 (4.97)
GF (2)	1	5.65 (2.47)	1.36 (0.59)	4.62 (1.21)
GF (2)	2	4.40 (0.93)	1.80 (0.57)	2.55 (0.45)

[a] Values are means for each group; standard deviations are given in parentheses.
[b] Significant ($P < 0.005$; two-sample t test).
[c] Significant ($P < 0.05$; two-sample t test).

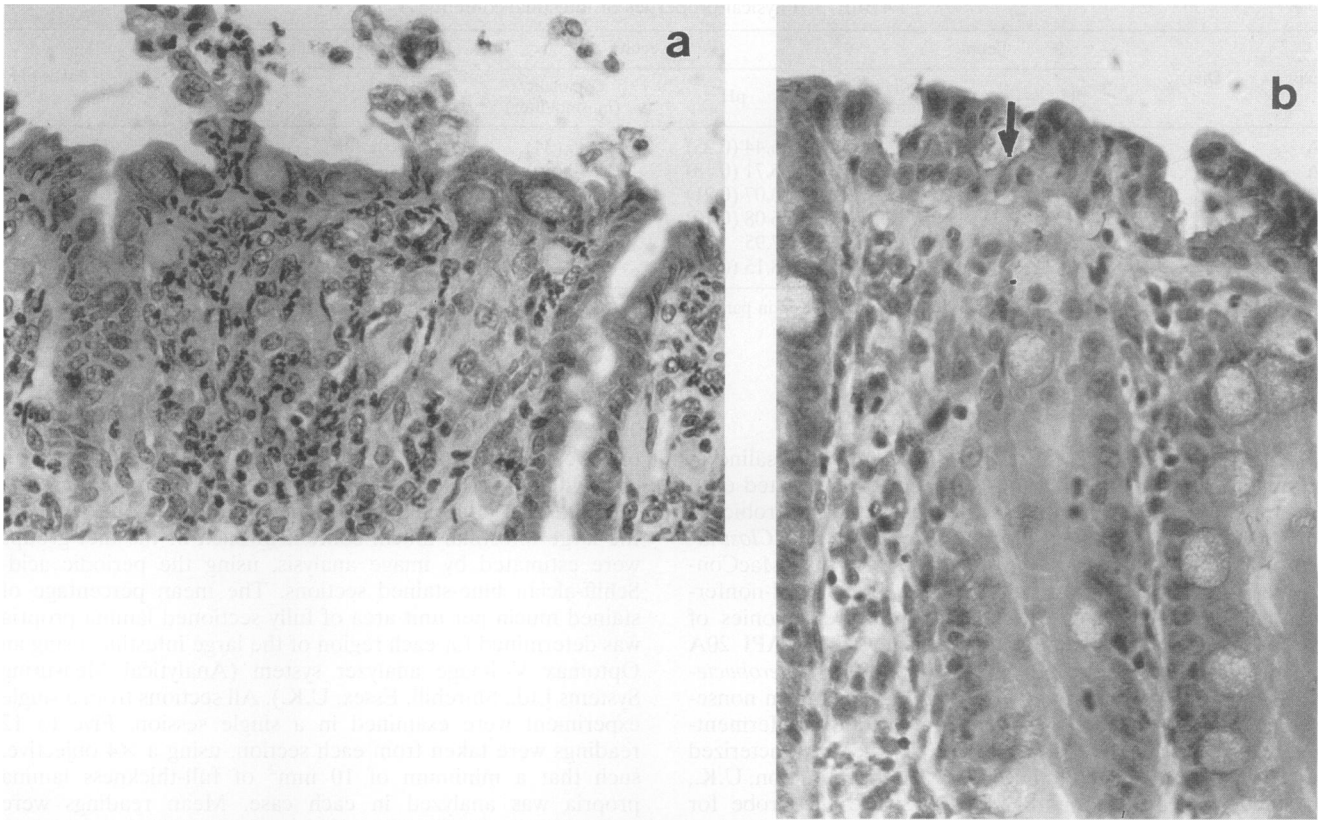

FIG. 1. Histological sections from the large intestine of artificially conventionalized pigs fed diet 2. (a) Epithelial cell loss with associated neutrophil infiltration of lamina propria and intestinal lumen. Hematoxylin and eosin stain was used. Magnification, ×300. (b) Focal areas of epithelial degeneration with associated adherent bacteria (arrow). Hematoxylin and eosin stain was used. Magnification, ×500. (c) Brightly stained coliform bacteria adherent to area of damaged epithelium. Immunoperoxidase stain and rabbit antisera to *E. coli* O116 at a 1:80 dilution were used. Magnification, ×700.

of the intestine for each experimental group was then calculated.

RESULTS

Clinical findings. No evidence of systemic illness was noted in any pig during the experimental period. "Loose" feces were observed from time to time in the diet 2 CV groups in experiment B. Total feed consumption amounts for the diet 1b group in experiment B were 8.9 and 8.8 kg for the periods 0 to 7 and 8 to 14 days postweaning, respectively. The corresponding values for the diet 2 group were 2.9 and 8.9 kg.

Postmortem findings. No intestinal lesions or other abnormalities were found in the GF pigs on either diet. In the CV pigs, no gross lesions were found in the large intestine of either group, except that the organ as a whole was rather congested and appeared to be about 50% larger in the pigs on diet 2 compared with those on diet 1b. Pigs were generally smaller in the diet 2 groups; body weight was significantly ($P < 0.05$) lower in experiment B (Table 2). The dry-matter contents of the feces of the two diet groups were similar in experiment A but significantly lower in the diet 2 group in experiment B. There were no significant differences between the physical parameters of pH or osmolality of intestinal content between the two diet groups, although in some cases these differed significantly between experiments A and B (Table 3). Peripheral leukocyte counts were elevated in the diet 2 groups in experiment A ($P < 0.005$) and also in experiment B ($P < 0.05$). This leukocytosis was accounted for by a neutrophilia (Table 4).

Histological sections. GF pigs (fed either diet 1 or diet 2) showed no histological abnormalities. Histological examination of the CV pig tissues showed multifocal epithelial degeneration and erosion, with a variable degree of associated neutrophil infiltration in all but one of the pigs on diet 2 in experiment A and in all pigs on diet 2 in experiment B. Gram-negative, rod-shaped bacteria were frequently seen associated with degenerative epithelium (Fig. 1). The lesions were always most severe in the large intestine, although the terminal ileum was affected in 5 of the 13 pigs on diet 2. Neutrophils were usually present at sites affected by this type of lesion, but the infiltration could be quite modest in some animals with extensive epithelial degeneration and, conversely, quite marked in animals with less severe lesions. There appeared to be a higher degree of neutrophil presence in the diet 2 group of experiment A compared with the corresponding group in experiment B. Total scores for the extent of the attaching and effacing lesions were highest in the cecum, proximal colon, and midcolon (Table 2). The large and small intestines of the diet 1b groups were essentially normal. In one case, some areas of epithelial cell flattening, associated with a mild neutrophil infiltration to the underlying lamina propria,

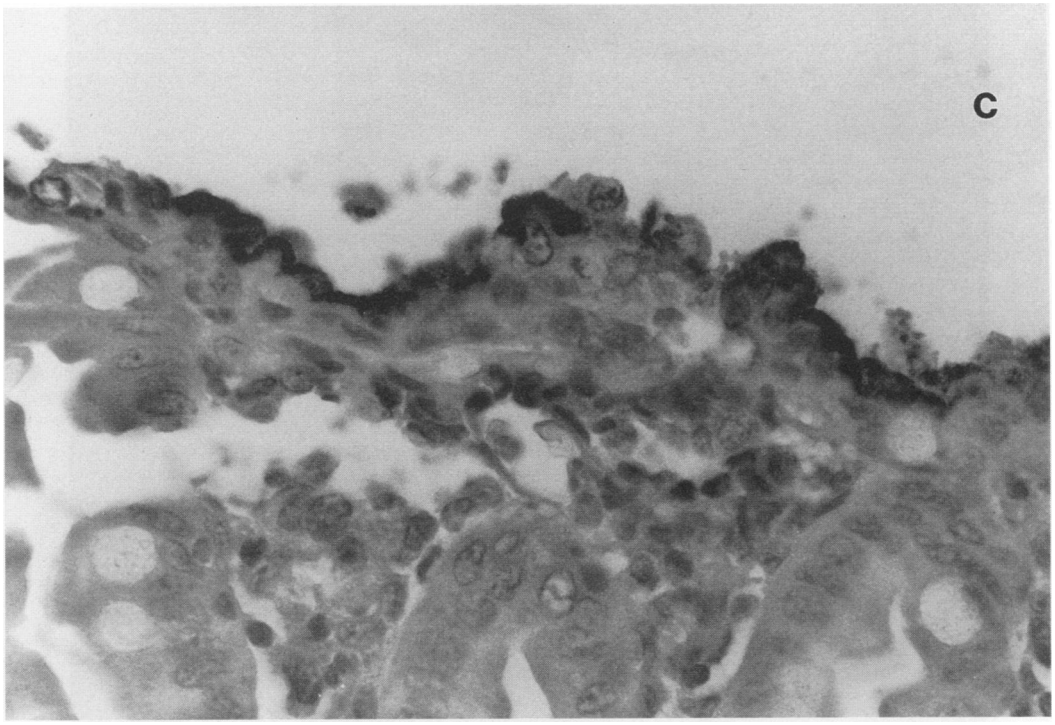

FIG. 1—*Continued.*

were found, but there was no accompanying attaching and effacing lesion.

In the immunoperoxidase assay incorporating antiserum to *E. coli* O116, brightly staining coliform bacteria were consistently seen in association with the degenerative epithelium in sections from affected colon of the CV pigs fed diet 2 (Fig. 1c). Stained bacteria were not observed in reactions with control *E. coli* antisera or in sections from pigs on the control diet (diet 1b).

Scanning electron microscopy showed no abnormalities present in the three animals examined from the diet 1b groups. All of the large intestinal tissue samples from the four sample pigs in the diet 2 groups showed rod-shaped bacteria adhering to the surface of enterocytes in a manner typical of attaching and effacing *E. coli* strains. Attachment was accompanied by a loss of microvilli from the surface of affected cells. Many enterocytes, particularly in the intercrypt regions, appeared to be sloughing (Fig. 2).

Crypt/villus ratios (Table 5) were significantly increased in the diet 2 groups in both experiment A and the GF experiment. They were very similar for the two diet groups in experiment B.

Bacteriology. Salmonellae, *Yersinia* spp., or organisms resembling spirochetes were not detected following bacteriological culture of CV fecal samples. Nonselective culture produced a moderate, mixed growth of a variety of colony types on both aerobic and anaerobic incubation in all cases.

In experiment B, in which more extensive bacteriology was performed, total fecal bacterial counts showed mean aerobic counts of 10^9 and 3×10^9 for diet 1b and diet 2 groups, respectively. Mean anaerobic counts were 2×10^9 and 2×10^{10}, respectively. Anaerobic species outnumbered aerobic species in all individual cases with the exception of one animal with soft feces in the diet 2 group. *Clostridium difficile* and *B. fragilis* were not recovered from any pigs. A sorbitol-nonfermenting *E. coli* O116 strain was recovered from three of the five pigs on diet 2 but not from any of the pigs on diet 1b. This strain was shown to possess the *eae* gene but not genes for either VT1 or VT2.

DISCUSSION

These experiments show that one or more AEEC strains, apparently present in low numbers as part of the commensal intestinal flora of asymptomatic animals, can play a role in the pathogenesis of a colitis that is induced by diet. Both the distribution and the nature of the lesions observed in these experiments were similar to those described for experimental infection of neonatal pigs with AEEC by other workers (27, 37, 39). The marked generalized congestion of the large intestine found in the diet 2 groups in these experiments has also been described as a feature of experimental infection of pigs with AEEC strains of human origin (39). The extent of neutrophil infiltration and the inflammatory response generally in the lesions might suggest an EPEC, rather than an enterohemorrhagic *E. coli*, attaching and effacing lesion (24, 38). This was further supported by the failure to isolate verocytotoxic *E. coli* from any of the pigs and the isolation of an *eae*-positive EPEC O116 strain from the majority of pigs in the tested diet 2 group (experiment B). All pigs in this group were affected by attaching and effacing lesions, in which *E. coli* O116 could be demonstrated. The severity of the inflammatory focus in the large intestine of the pigs in both diet 2 groups was reflected in the marked peripheral neutrophilia observed in most animals.

The AEEC strain responsible for the lesions were almost certainly present in the original enteric flora that was used to colonize all of the pigs. Both diet 1 and diet 2 had been sterilized by irradiation, and there was no bacterial colonization or lesions apparent in the GF pigs fed either of the diets. Diet 2 itself, therefore, could not have been the source of the attaching and effacing bacterial strain. It is unlikely that

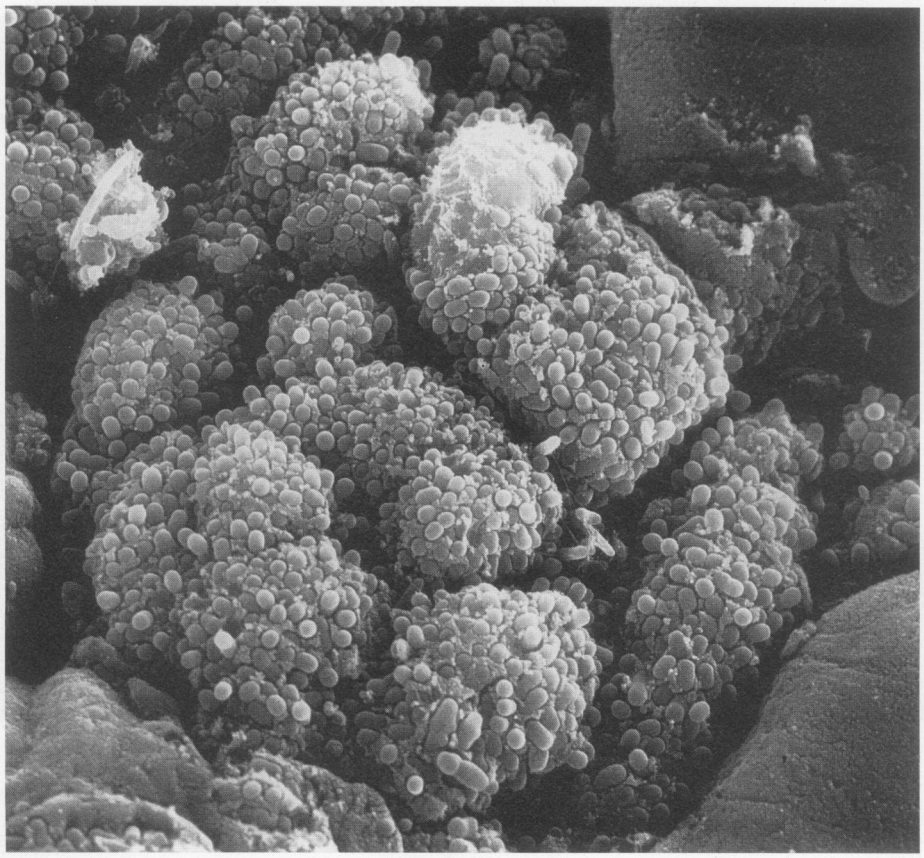

FIG. 2. Scanning electron micrograph of colon section of pig fed diet 2 showing extensive bacterial attachment with effacement of surface villi. Magnification, ×3,660.

accidental infection with the pathogen occurred in the diet 2 groups but not the diet 1b groups since the experiment was repeated with the same results several months later and in different animal accommodation from that used in the first instance.

Since an EPEC strain was demonstrated in lesions and recovered from the majority of tested pigs on diet 2, but not demonstrated or recovered from the diet 1b group, it seems likely that a major factor in this dietary-bacterial interaction was the selective proliferation of the EPEC strain, *E. coli* O116. It is also possible that the intestinal environment provided by diet 2 was able to influence the expression of pathogenicity of *E. coli* O116, rather than simply allowing its selective proliferation. Selective proliferation of AEEC could have occurred as a result of a direct effect of the ileal input to the large intestine satisfying a nutritional requirement of the AEEC strain itself. A more likely explanation is that alterations in the balance of the complex intestinal microflora brought about by this particular diet resulted in a reduction in the colonization resistance provided by the commensal microflora to the proliferation of this AEEC strain. There is evidence of colonization resistance by a normal intestinal microflora to AEEC strains in other species. Colonization of newly hatched chicks by the human AEEC strain O157:H7 is significantly reduced by prior administration of an adult-type microflora (34).

TABLE 5. Terminal ileal crypt/villus ratios and large-intestinal goblet cell mucin estimates[a]

Expt	Diet	Ileal crypt/villus ratio	% of area of lamina propria stained with periodic acid-Schiff-alcian blue			
			Cecum	Proximal colon	Midcolon	Distal colon
A	1b	0.55 (0.16)	2.9 (1.7)	9.1 (5.7)	13.3 (4.1)	13.0 (6.2)
A	2	0.80 (0.17)[b]	0.9 (0.7)[b]	2.7 (3.1)[b]	7.0 (5.5)[b]	11.1 (7.0)
B	1b	0.54 (0.09)	5.7 (2.8)	10.4 (4.5)	14.94 (4.8)	11.0 (4.3)
B	2	0.60 (0.13)	1.9 (1.5)[b]	1.8 (2.4)[b]	6.6 (5.0)[b]	8.8 (4.7)
GF	1	0.2 (0.04)	2.5 (1.1)	5.1 (2.6)	2.4 (2.8)	1.8 (2.0)
GF	2	0.38 (0.07)	4.5 (4.0)	8.8 (5.6)	9.4 (9.1)	8.5 (8.7)

[a] Values are means for each group; standard deviations are given in parentheses.
[b] Significant ($P < 0.05$; two-sample t test).

The bovine enteric flora used to colonize these pigs was chosen to provide a fermentative gut flora free of known pig pathogens for study of the effects of certain diets on the large intestine of weaned pigs. It resembled the normal fecal flora of humans and a variety of weaned domestic species in that there were many more anaerobes than facultative bacteria present. AEEC have been isolated on numerous occasions from both healthy adult bovines and milk-fed calves with diarrhea (14, 19). The likely presence of an AEEC strain in the bovine flora used here is not, therefore, particularly remarkable. Recorded isolations of AEEC from swine are less common, but lack of routinely available diagnostic tests for these bacteria may have resulted in few isolates being identified as AEEC. Helie et al.

(16) isolated EPEC serotype O45:KE"65" from 3% of diarrheic pig fecal samples submitted to a diagnostic laboratory but identified the strain by serotype, with subsequent confirmation of its attaching and effacing properties. Thus, it is possible that AEEC may be present in swine more commonly than is generally supposed and that the diet-associated colitis seen in the experimental pigs used here may not be solely a phenomenon associated with the use of a bovine flora but could represent a naturally occurring form of porcine colitis.

Although AEEC-associated diarrhea has been reproduced in milk-fed neonatal conventional pigs (37), the bovine flora used in the CV pigs described here did not cause clinical symptoms in milk-fed calves or pigs used for its passage prior to these experiments. Weaning to solid food did not in itself precipitate the development of attaching and effacing lesions, since lesions were absent from the group weaned onto diet 1b. It is possible that nonspecific bactericidal agents found in milk, such as lactoferrin and lactoperoxidase (32), accounted for the suppression of proliferation of the EPEC strain O116 in the pigs fed either a liquid milk diet or diet 1b (a milk-based solid feed with an additional source of dietary fiber). In another study, however, a change from a low-protein, non-milk-based, high-fiber diet to a high-protein diet led to increased susceptibility to enterotoxigenic *E. coli*-induced diarrhea (32), suggesting that in the case of that strain of *E. coli*, at least, the bactericidal properties of milk were not important in the suppression of proliferation. In other studies, in which pigs colonized with the same bovine microflora used here were weaned onto other, non-milk-based diets that were not associated with diarrhea in the field, no attaching and effacing lesions or clinical symptoms were observed (29). Milk may not, therefore, be an essential component of a diet that does not permit the proliferation of the O116 EPEC strain apparently present at low levels in the commensal flora of the pigs used in these experiments.

Although there was severe physical damage in the large intestine of many of the conventional pigs, and the fecal dry-matter content was reduced in experiment B, the pathogenic effects of these lesions were insufficient to result in a reduction of fecal dry-matter content below 20%, a level suggested as constituting "diarrhea" (23). It has been noted that many AEEC strains isolated from humans with severe diarrhea produce relatively mild clinical effects in neonatal gnotobiotic and conventional pig models (27). One explanation offered for this is that AEEC lesions occur predominantly in the colon, while the liquid milk diet fed in restricted quantities to these very young animals is well digested and absorbed in the small intestine, leaving minimal quantities of solute and water for absorption by the large intestine. The clinical effects of physical damage to the large intestine were therefore thought likely to be mild (27). Similarly, certain AEEC strains isolated from rabbits have been observed to produce AEEC lesions but not diarrhea in unweaned rabbits. Again, the relatively low contribution of the large intestine to food absorption in milk-fed unweaned animals, compared with weaned animals on a cereal-based diet that requires some large-intestinal digestion, was suggested as a reason for the absence of clinical signs in the unweaned groups (31). The experiments described here, however, showed that even quite severe lesions in some cases did not lead to diarrhea in weaned animals on a cereal-type diet (diet 2), which is unlikely to be completely digested in the small intestine. This type of diet (3.5% crude fiber) would be likely to present the large intestine with a relative absorptive load at least equivalent to that presented to the large intestine of an adult human. Thus, the fact that diarrhea did not occur in the experimental pigs with severe AEEC lesions described here suggests that the hypothesis that severe AEEC diarrheas result principally from the loss of large intestinal absorptive epithelium subsequent to attaching and effacing lesions (27) may not represent the entire pathogenesis in some of the more severe human cases. When AEEC strains have been suspected as the pathogenic agent responsible in field cases of diarrhea in pigs, other potential pathogens were also isolated (18). These, rather than the AEEC, may have been the cause of the clinically apparent diarrhea in the cases cited.

Porcine colitis syndrome is a poorly characterized diarrheal disease that appears to be strongly influenced by diet changes (36). This experimental model of a diet-induced colitis suggests that some diet-responsive cases of porcine colitis may be mediated by the activities of AEEC. It is also possible that in the presence of an adult-type intestinal flora the development of this type of lesion (with or without clinical signs) in species other than the pig may be strongly affected by diet. This in turn has implications for the use of milk-fed animals as models for examining the pathogenicity of *E. coli* strains recovered from adult humans or domestic animals on nonmilk diets who are suffering from diarrhea.

ACKNOWLEDGMENTS

We acknowledge the Ministry of Agriculture, Fisheries and Food for funding this work; Henry Smith of the Public Health Laboratory for advice and characterization of *E. coli* isolates; J. H. Morgan for use of the bovine intestinal flora; and S. Hacker and H. Cook for staining histology specimens.

REFERENCES

1. **Argenzio, R. A., and D. J. Meuten.** 1991. Short-chain fatty acids induce reversible injury of the porcine colon. Dig. Dis. Sci. **36**:1459–1468.
2. **Argenzio, R. A., and S. C. Whipp.** 1980. Pathophysiology of diarrhea, p. 220–232. *In* N. V. Anderson (ed.), Veterinary gastroenterology. Lea and Febiger, Philadelphia.
3. **Aspinall, S. T., and D. N. Hutchinson.** 1992. New selective medium for isolating *Clostridium difficile* from faeces. J. Clin. Pathol. **45**:812–814.
4. **Bertschinger, H. U., E. Eggenburger, H. Jucker, and H. P. Pfirter.** 1979. Evaluation of low-nutrient, high-fibre diets for the prevention of *Escherichia coli* enterotoxaemia. Vet. Microbiol. **3**:281–290.
5. **Border, M., B. D. Firehammer, D. S. Shoop, and L. L. Myers.** 1985. Isolation of *Bacteroides fragilis* from diarrheic calves and lambs. J. Clin. Microbiol. **21**:472–473.
6. **Cantey, J. R., and R. K. Blake.** 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. J. Infect. Dis. **135**:454–462.
7. **Cohen, M. B., and R. A. Gaginella.** 1991. Bacterial infections—pathophysiology, clinical features and treatment, p. 394–428 *In* S. F. Phillips et al. (ed.), The large intestine: physiology, pathophysiology and disease. Raven Press, New York.
8. **Collins, J. E., M. E. Bergeland, L. L. Myers, and D. S. Shoop.** 1989. Exfoliating colitis associated with enterotoxigenic *Bacteroides fragilis* in a piglet. J. Vet. Diagn. Invest. **1**:349–351.
9. **Coussement, W., R. Ducatelle, G. Charlier, L. Okerman, and J. Hoorens.** 1984. Pathology of experimental colibacillosis in rabbits. Zentralbl. Veterinaer Med. Reihe B **31**:64–72.
10. **Danielson, D. M., and J. D. Crenshaw.** 1991. Raw and processed soybeans in swine, p. 573–583. *In* E. R. Miller and D. E. Ullrey (ed.), Swine nutrition. Butterworth-Heinemann, Stoneham, Mass.
11. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60**:3953–3961.
12. **Duncan, A. L., and R. J. Lysons.** 1987. Diagnosis of colitis in pigs. Vet. Rec. **121**:430.
13. **Hall, G. A., N. Chanter, and A. P. Bland.** 1988. Comparison in gnotobiotic pigs of lesions caused by verotoxigenic and non-verotoxigenic *Escherichia coli*. Vet. Pathol. **25**:205–210.
14. **Hall, G. A., D. J. Reynolds, N. Chanter, J. H. Morgan, K. R.**

Parsons, T. G. Debney, A. P. Bland, and J. C. Bridger. 1985. Dysentery caused by *Escherichia coli* S102-9 in calves: natural and experimental disease. Vet. Pathol. **22:**156–163.
15. **Hazzledine, M. J.** 1987. Unresolved problems—nutrition and disease. Pig Vet. J. **19:**79–90.
16. **Helie, P., M. Morin, M. Jacques, and J. M. Fairbrother.** 1991. Experimental infection of newborn pigs with an attaching and effacing *Escherichia coli* O45:K"E65" strain. Infect. Immun. **59:** 814–821.
17. **Heppel, L. M. J., J. W. Sissons, and S. M. Banks.** 1989. Sensitisation of preruminant calves and pigs to antigenic protein in early weaning diets: control of the systemic antibody responses. Res. Vet. Sci. **47:**257–262.
18. **Janke, B. H., D. H. Francis, J. E. Collins, M. C. Libal, D. H. Zeman, and D. D. Johnson.** 1989. Attaching and effacing *Escherichia coli* infections in calves, pigs, lambs and dogs. J. Vet. Diagn. Invest. **1:**6–11.
19. **Janke, B. H., D. H. Francis, J. E. Collins, M. C. Libal, D. H. Zeman, D. D. Johnson, and R. D. Neiger.** 1990. Attaching and effacing *Escherichia coli* as a cause of diarrhea in young calves. J. Am. Vet. Med. Assoc. **196:**897–901.
20. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1987. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
21. **Johnson, E., and S. W. Barthold.** 1979. The ultrastructure of transmissible murine colonic hyperplasia. Am. J. Pathol. **97:**291–313.
22. **Jones, M. A., and D. Hunter.** 1983. Isolation of *Clostridium difficile* from pigs. Vet. Rec. **112:**253.
23. **Kenworthy, R., and W. D. Allen.** 1966. The significance of *Escherichia coli* to the young pig. J. Comp. Pathol. **76:**31–44.
24. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea. Enterotoxic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J. Infect. Dis. **155:**377–389.
25. **Lysons, R. J., G. A. Hall, R. M. Lemcke, J. Bew, and P. D. Luther.** 1980. Studies of organisms possibly implicated in swine dysentery, p. 231. *In* Proceedings of the International Pig Veterinary Society 1980, Copenhagen, Denmark.
26. **Martin, S. R., and W. A. Walker.** 1991. Response of the colon to injury: inflammation, immunity and mucus. *In* S. F. Phillips et al. (ed.), The large intestine: physiology, pathophysiology and disease. Raven Press, New York.
27. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Gianella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41:**1340–1351.
28. **Morgan, J. H.** 1990. Ph.D. thesis. University of Reading, Reading, United Kingdom.
29. **Neef, N. A., R. J. Lysons, D. J. Trott, D. J. Hampson, P. W. Jones, and J. H. Morgan.** 1994. Pathogenicity of porcine intestinal spirochetes in gnotobiotic pigs. Infect. Immun. **62:**2395–2403.
30. **Neutra, M. R., and J. F. Forstner.** 1987. Gastrointestinal mucus: synthesis, secretion and function, p. 975–1009. *In* L. R. Johnson (ed.), Physiology of the gastrointestinal tract, 2nd ed. Raven Press, New York.
31. **Peeters, J. E., G. J. Charlier, and P. H. Halen.** 1984. Pathogenicity of attaching and effacing *Escherichia coli* isolated from diarrheic suckling and weanling rabbits or newborn rabbits. Infect. Immun. **46:**690–696.
32. **Reiter, B.** 1978. Review of the progress of dairy science: antimicrobial systems in milk. J. Dairy Res. **45:**131–148.
33. **Smith, W. J., and E. P. Nelson.** 1987. Grower scour/non-specific colitis. Vet. Rec. **121:**334.
34. **Stavric, S., B. Buchanan, and T. M. Gleeson.** 1992. Competitive exclusion of *Escherichia coli* O157:H7 from chicks with anaerobic cultures of faecal microflora. Lett. Appl. Microbiol. **14:**191–193.
35. **Tavernor, W. D., P. C. Trexler, L. C. Vaughan, J. E. Cox, and D. G. C. Jones.** 1971. The production of gnotobiotic pigs and calves by hysterotomy under general anaesthesia. Vet. Rec. **88:**10–14.
36. **Taylor, D. J.** 1989. Pig diseases, 5th ed., p. 269. Burlington Press, Cambridge.
37. **Tzipori, S., R. Gibson, and J. Montanaro.** 1989. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. Infect. Immun. **57:**1142–1150.
38. **Tzipori, S., H. Karch, K. I. Wachsmuth, R. M. Robins-Browne, A. D. O'Brien, H. Lior, M. L. Cohen, J. Smithers, and M. M. Levine.** 1987. Role of a 60-megadalton plasmid and shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. **55:**3117–3125.
39. **Tzipori, S., K. I. Wachsmuth, J. Smithers, and C. Jackson.** 1988. Studies in gnotobiotic piglets on non-O157:H7 *Escherichia coli* serotypes isolated from patients with hemorrhagic colitis. Gastroenterology **94:**590–597.
40. **Wilkinson, J. D., and E. N. Wood.** 1987. Grower scour/non-specific colitis. Vet. Rec. **121:**406.
41. **Willshaw, G. A., H. R. Smith, S. M. Scotland, A. M. Field, and B. Rowe.** 1990. Heterogeneity of *Escherichia coli* phages encoding Vero cytotoxins: comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J. Gen. Microbiol. **133:**1309–1317.