

Colonization, Persistence, and Tissue Tropism of *Escherichia coli* O26 in Conventionally Reared Weaned Lambs[∇]

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Escherichia coli O26 is recognized as an emerging pathogen associated with disease in both ruminants and humans. Compared to those of *E. coli* O157:H7, the shedding pattern and location of *E. coli* O26 in the gastrointestinal tract (GIT) of ruminants are poorly understood. In the studies reported here, an *stx*-negative *E. coli* O26 strain of ovine origin was inoculated orally into 6-week-old lambs and the shedding pattern of the O26 strain was monitored by serial bacteriological examination of feces. The location of colonization in the GIT was examined at necropsy at two time points. The numbers of O26 organisms excreted in feces declined from approximately 10⁷ to 10⁴ CFU per gram of feces by day 7 and continued at this level for a further 3 weeks. Beyond day 30, excretion was from few animals, intermittent, and just above the detection limit. By day 38, all fecal samples were negative, but at necropsy, O26 organisms were recovered from the upper GIT, specifically the ileum. However, no attaching-effacing (AE) lesions were observed. To identify the location of *E. coli* O26 within the GIT early after inoculation, two lambs were examined postmortem, 4 days postinoculation. High numbers of O26 organisms were recovered from all GIT sites examined, and ~10⁹ CFU were recovered from 1 gram of ileal tissue from one animal. Despite high numbers of O26 organisms, AE lesions were identified on the mucosa of the ascending colon of only one animal. These data indicate that *E. coli* O26 readily colonizes 6-week-old lambs, but the sparseness of AE lesions suggests that O26 is well adapted to this host, and mechanisms other than those dependent upon intimin may play a role in persistence.

Attaching-effacing *Escherichia coli* (AEEC) strains associated with human gastrointestinal disease are classified as either enteropathogenic *E. coli* (EPEC) or enterohemorrhagic *E. coli* (EHEC), depending on their abilities to produce Shiga toxins (41). Although *E. coli* O157:H7 is the most prevalent EHEC serotype associated with serious disease in humans, non-O157:H7 EHEC strains represent an emerging threat to public health and, unlike O157 strains, to animal health also (24). Indeed, non-O157 EHEC may be more prevalent than serogroup O157 in some countries (3).

E. coli O26 is a well-recognized EPEC serogroup associated with disease in humans, notably infantile diarrhea (36). Significantly, Shiga toxin-elaborating *E. coli* (STEC) O26 bacteria are also associated with serious human infections, including severe diarrhea with sequelae similar to those of EHEC infections. Such infections have been recorded in Italy (55), Ireland (38), Japan (28, 29), Scotland (51), and elsewhere. The World Health Organization (WHO) has identified O26 STEC as the second most important serogroup of *E. coli* (65).

EHEC and EPEC belong to the AEEC pathotype whose bacteria attach intimately to the microvillus brush border of enterocytes of animals and humans, forming attaching-effacing (AE) lesions (47, 57). The genes encoding the classical AE histopathology are located on a pathogenicity island known as the locus of enterocyte effacement (LEE) (37). Intimin, en-

coded by the *eae* gene within the LEE, is one major effector protein required for intimate attachment and AE lesion formation by EPEC (17) and EHEC (56). AEEC strains are prevalent in cattle (19, 49) and sheep (4, 9), and intimin has been shown to be an important factor for colonization and AE lesion formation in animals (12, 66). AEEC O26-associated AE lesions were observed in the large intestine of an 8-month-old heifer with diarrhea (45) and incidentally during experiments involving the inoculation of sheep with *E. coli* O157:H7 (61). Different intimin types may play a role in determining the pattern of colonization and tissue tropism in the host (20). *E. coli* O157 produces γ -intimin and is regarded to have a tropism toward the distal gastrointestinal tract (GIT), while *E. coli* O26 produces β -intimin and is regarded to have a tropism toward the proximal GIT (8).

E. coli O157:H7 has been experimentally inoculated into cattle, calves, sheep, and lambs (6, 10, 11, 31), and the distal GIT, including the cecum, colon, and rectum, is the principal site of *E. coli* O157:H7 colonization in both species (6, 13, 14, 15, 25, 59). The studies of Naylor et al. (42) indicate that *E. coli* O157 may exhibit a tissue tropism for the terminal rectum in experimentally infected calves. In calves, serogroups O5 and O111 adhere to the colonic epithelium preferentially (54) and STEC O26:H– adhered in large numbers to the colonic, cecal, and rectal mucosa (58). AE lesion formation by STEC O26:K60:H11 was also observed in the spiral colons of 6-month-old sheep in a ligated-loop model (60).

Studies on Scottish beef farms have shown that 94% of calves shed *E. coli* O26, with over 90% of individual strains carrying virulence genes associated with human disease, such as the *stx*, *eae*, and *hlyA* genes (44, 52). In a recent abattoir

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study conducted in England and Wales, we showed that *E. coli* O26 bacteria are prevalent in ruminants, particularly sheep (1). Given that the prevalence of serogroup O26 in ruminants is high (5, 64) and the threat to public health may be significant (8, 38, 40), it was considered that a greater understanding of the shedding pattern and the site of colonization of *E. coli* O26 in sheep should be gained. Here, we report the use of three models, namely, the ovine ligated-intestinal-loop model, the ovine intestinal in vitro organ culture (IVOC) model, and the 6-week-old-lamb oral inoculation model, to investigate this.

MATERIALS AND METHODS

Animals. All animal studies were performed in accordance with the Animals (Scientific Procedures) Act (1986) and were approved by the local Ethical Review Committee. All animals used in these studies were screened for excretion of *E. coli* O157 and O26 serogroups prior to any in vitro or in vivo studies by immunomagnetic bead separation (IMS) as described below.

Bacterial strains and preparation of inocula. *E. coli* O26:K60 strains EC740/01, EC746/01, EC417/02, EC460/02, and EC335/98 were of sheep origins and were obtained from the Veterinary Laboratories Agency (VLA; Weybridge, United Kingdom) culture collection. These strains lack the *stx*₁ and *stx*₂ toxin genes but possess the β 1-intimin gene. Other than strain EC335/98, which is naturally resistant (>250 μ g/ml) to streptomycin (Str^r) only, all strains were fully sensitive to the antibiotics ($n = 16$) tested (VLA reference laboratory procedure). The *E. coli* O157:H7 NCTC12900 NaI^r strain lacks the *stx*₁ and *stx*₂ toxin genes, possesses the γ -intimin gene, and has been fully characterized previously (16, 32).

All strains were stored at -80°C in heart infusion broth supplemented with 30% glycerol and maintained on Dorset egg slopes at an ambient temperature for experimental work. To prepare cultures for use, strains were streaked on 5% sheep blood agar to give single colonies when required and then grown overnight in Luria-Bertani (LB) broth for 16 h at 37°C with shaking (225 rpm) to give $\sim 1 \times 10^9$ CFU ml⁻¹. Bacterial cultures were centrifuged for 10 min at 4,800 rpm, and the pellet was resuspended to give the desired concentration in phosphate-buffered saline (PBS; 0.1 M, pH 7.2) or Dulbecco's modified Eagle's medium (DMEM; Sigma) as required.

For adhesion and invasion assays and for in vitro organ culture association assays, the bacterial inocula were prepared in PBS and the strains were resuspended to give 1×10^9 CFU ml⁻¹, whereas for the ovine ligated-loop assays, the strains were resuspended in DMEM supplemented with 1% (vol/vol) nonessential amino acids (Sigma) and 1% (vol/vol) L-glutamine (Sigma) to give 1×10^9 CFU ml⁻¹. For in vivo studies, the strains were resuspended in PBS to give 1×10^9 CFU ml⁻¹ and the inoculum comprising 10 ml was delivered to the pharynx using a conventional dosing gun.

Recovery and enumeration of bacterial strains from in vitro and in vivo assays. Unless stated otherwise, all dilution series for direct counting were plated on sorbitol MacConkey (SMAC; Oxoid, Basingstoke, United Kingdom) agar plates supplemented when appropriate with streptomycin (250 μ g ml⁻¹) or nalidixic acid (15 μ g ml⁻¹). Luminal content and tissue samples were weighed to 1 g, resuspended in 9 ml of buffered peptone water, mashed with sterile forceps, and vortexed, and 10-fold serial dilutions were plated directly. The recovery of O26 and O157 strains from feces was done by the use of anti-*E. coli* O26 and O157 Dynabeads (Dyna; Oslo, Norway) in an automated BeadRetriever (Dyna; Oslo, Norway), as directed by the user manual. One hundred microliters of the final washed bead suspensions was streaked onto SMAC agar, and single colonies were tested for agglutination. For presumptive non-sorbitol-fermenting O157 colonies, a portion of a single colony was emulsified in a drop of saline on a reaction card to which one drop of O157-specific latex (*E. coli* O157:H7 Test; Oxoid) was added. The card was rocked for up to a minute for the observation of agglutination. For presumptive sorbitol-fermenting O26 colonies, the same approach was applied, except O26:K60-specific capsular antisera prepared at the VLA was used to test for agglutination. If no direct counts were observed for IMS, buffered peptone water homogenates and dilutions were enriched by incubation at 37°C for 6 h and then plated onto SMAC agar plates supplemented with the appropriate antibiotics.

To assess the numbers of background aerobic flora from tissues, the dilution series were plated on plate count agar without the addition of any selective antibiotics.

Bacterial adherence and invasion assays, Giemsa staining, FAS, scanning electron microscopy, and TEM. Bacterial adherence and invasion assay, Giemsa staining, fluorescent actin staining (FAS) and scanning and transmission electron

microscopy (TEM) were performed essentially as previously described (1, 35). HEP-2 and Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, United Kingdom) and treated similarly. Cells were cultured at 37°C (5% CO₂) in DMEM (Sigma) supplemented with 10% fetal bovine serum (Autogen Bioclear). Subconfluent cultures were split 1:3 to 1:6 (seeding at about 2×10^4 to 4×10^4 cells/cm²) using 0.25% trypsin-EDTA (Sigma). Cells were routinely passaged in 75-cm² tissue culture flasks (Nunc) and set up for experimental use in multiwell plates (Nunc). During culture, cells were always passaged before reaching confluence (every 4 to 6 days) to minimize cytoplasmic vacuolation and care was needed to disperse cell clumps into a single cell suspension when split. Media in experimental plates were changed every 4 to 6 days until the plates were required for use. Undifferentiated cells were grown to confluence in 48 h. For TEM, differentiated cells were grown for 15 days.

Ovine GIT in vitro organ culture association assay. IVOC was based on methods described previously for bovine, pig, and human IVOC assays (2, 27, 46). Briefly, approximately 2-cm² tissue samples were obtained from the GITs of the lambs that were euthanized by captive bolt, followed by exsanguination. Tissue samples included those from the duodenum, the jejunum, the ileum, the ascending colon, the spiral colon, the cecum, the middle region of the rectum, and the rectal-anal junction (RAJ). All tissue samples were placed into Duran bottles containing 95 ml of DMEM (1% nonessential amino acids and 1% L-glutamine). A bacterial inoculum of 5 ml containing *E. coli* O157 or O26 bacteria was added to each Duran bottle. The bottles were then incubated at 37°C (5% CO₂) for 6 h, with the culture medium being completely replaced every 2 h. After incubation, each tissue sample was washed three times with Hanks balanced salt solution. Each tissue sample was then disrupted for 10 min by using a solution of 1% Triton X-100 (Sigma) and vigorous shaking.

Ovine ligated-loop model. One 6-month-old cross-bred lamb was used. The procedures used were essentially as described previously (60).

In vivo lamb experiments. An ovine oral inoculation model was used as described previously (63) with minor modifications. A total of six 6-week-old conventional cross-bred lambs were orally challenged with $\sim 10^{10}$ CFU of EC335/98 just before morning feeding. Rectal fecal samples were taken from all animals daily for the first 17 days and on days 20, 21, 22, 23, 24, 27, 28, 29, 30, 31, 34, 36, and 38 postinoculation (p.i.). On day 38, when the study was terminated, three lambs were euthanized by intravenous overdoses of barbiturate (Somulose). Tissue samples included those from the rumen, the duodenum, the jejunum, the ileum (six sections of the ileum were taken, starting approximately 5 cm from the ileo-cecal junction), the ascending colon, the spiral colon, the cecum, the rectum (six sections of the rectum were taken, starting approximately 2 cm from the RAJ), and the RAJ. The tissue samples were collected from three lambs and dissected to give a 1-g sample for bacteriological analysis as described above.

On a separate occasion, two 6-week-old conventional cross-bred lambs were orally challenged with $\sim 10^{10}$ CFU of EC335/98 as controls as described above and rectal fecal samples were taken daily. Both lambs were euthanized by intravenous injection of barbiturate (Somulose) on day 4 p.i. The rest of the procedure was as described above.

Pathological studies. Light and electron microscopy and immunohistochemistry were performed essentially as described previously (32, 33, 63). Briefly, tissues were fixed in 10% neutral buffered Formalin, processed to wax, and sectioned at 4 μ m. Sections were rehydrated prior to assembly into a Shandon Sequenza staining apparatus. The slides were washed with 2 \times sodium chloride-Tris-buffered saline (TBS) (0.005 M TBS, pH 7.6; 1.7% NaCl) for 5 min before incubation at room temperature with a normal goat serum (Vector Labs, United Kingdom). *E. coli* O26 or O157 polyclonal antibodies, raised in rabbits (VLA, Weybridge, United Kingdom), were then applied (1/1,000 and 1/5,000 diluted in 2 \times NaCl-TBS supplemented with 5% normal rabbit serum). *E. coli* antigens were visualized through incubation with biotinylated goat anti-rabbit immunoglobulin G. The sections were then counterstained in Meyers's hematoxylin.

Statistical analysis. For statistical analyses of assays, counts were translated to log₁₀ values, one being added if there were zero counts, and analyses of variance performed. The model for the analysis of the ovine GIT in vitro organ culture association assay included the main effects of the gut section and the strain and their interaction. The strains were compared to the control and to each other by *t* tests, with the Bonferroni adjustment applied to the *P* values.

RESULTS

Adhesion and invasion of *E. coli* O26 with respect to human HEP-2 and Caco-2 cells. There were no significant differences in the numbers of O26 organisms that adhered to HEP-2 cells compared to what was found for O157 ($P = 0.552$), whereas for

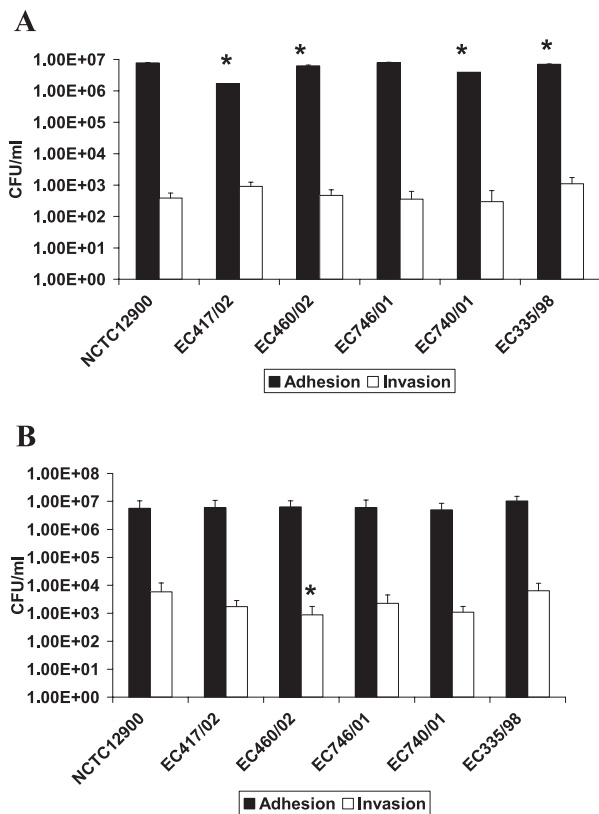


FIG. 1. (A) Adhesion (black) and invasion (white) of O26 strains with respect to Caco-2 tissue culture cells. (B) Adhesion (black) and invasion (white) of O26 strains with respect to HEp-2 tissue culture cells. NCTC12900 was used as a control strain for both cell lines. Results are presented as the means \pm standard deviations from the means. Asterisks indicate strains for which the numbers of organisms adhered to/invaded were statistically significant ($P < 0.05$) compared to what was found for NCTC12900.

invasion, significant differences were seen with strain EC460/02 ($P = 0.0041$). Significant differences were also seen with strains EC335/98, EC740/01, EC460/02, and EC417/02 ($P = 0.001$) adhering to Caco-2 cell lines, whereas for invasion, there were no significant differences compared to what was found for O157 (Fig. 1A and B).

The adherence pattern of the O26 strains was determined by light microscopy and scored after 6 h incubation of the bacteria associated with HEp-2 and Caco-2 cells. All O26 strains induced FAS-positive reactions on HEp-2 cells, and the pattern of adherent cells was of well-developed microcolonies, typical for intimately attached AEEC strains (Fig. 2). All strains were found to induce AE lesions on both cell lines when observed by TEM (Fig. 3).

Selection of *E. coli* O26 EC335/98 for in vivo studies. Nalidixic acid- and streptomycin-resistant derivatives of the O26 strains were made by plating the strains on gradient plates to enable easier recovery from in vivo experiments. All nalidixic acid-resistant derivatives were attenuated with respect to adherence and FAS, as were the four streptomycin-resistant derivatives (data not shown). Thus, strain EC335/98, which was a naturally occurring streptomycin-resistant O26 strain from sheep, was selected for in vivo studies.

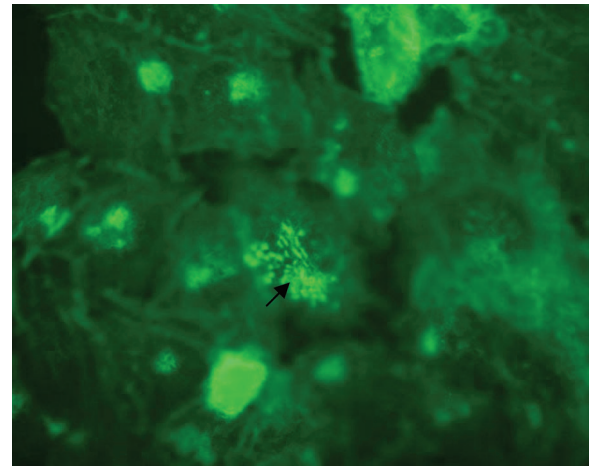


FIG. 2. *E. coli* O26-induced actin accumulation in HEp-2 cells. After *E. coli* O26 was incubated with tissue culture monolayers for 6 h, the cells were permeabilized with 0.1% Triton X-100 and then stained with fluorescein isothiocyanate-phalloidin. Monolayers were viewed with a fluorescence microscope in order to detect actin accumulation in tissue culture cells. Magnification, $\times 1,000$.

Interaction of the *E. coli* O26 EC335/98 strain with ovine IVOC. The highest numbers of adhering O26 bacteria were recovered from the ascending colon, and the lowest numbers of adhering bacteria were recovered from the ileum (Fig. 4). However, none of these differences were significant. The numbers of aerobic background bacteria were also determined for these samples and shown to be in the order of 2 \log_{10} lower than those for either O26 or O157 ($P < 0.0001$) in all tissues. No tissue sections were taken for histological examination.

Colonization and persistence of *E. coli* O26 EC335/98 in 6-week-old lambs. All lambs were clinically normal, healthy, and free from disease at the onset of the study. None were infected by O26 or O157 as tested by IMS of multiple fecal samples. Additionally, no streptomycin resistance colonies were formed when fecal samples were plated on the medium selecting for EC335/98. After oral inoculation of the lambs with 10^{10} CFU ml^{-1} of EC335/98 bacteria, no clinical signs or

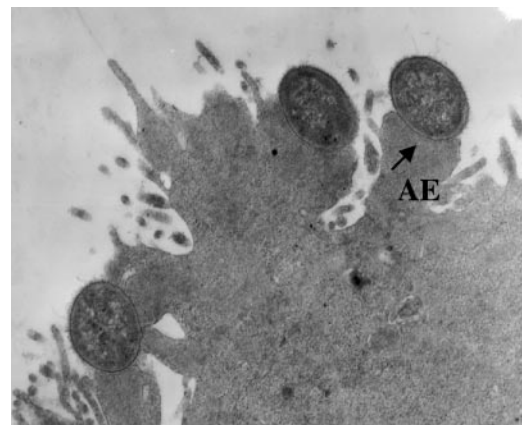


FIG. 3. Transmission electron micrograph showing strain EC335/98-infected HEp-2 cells with AE lesion formation (arrow). Magnification, $\times 35,000$.

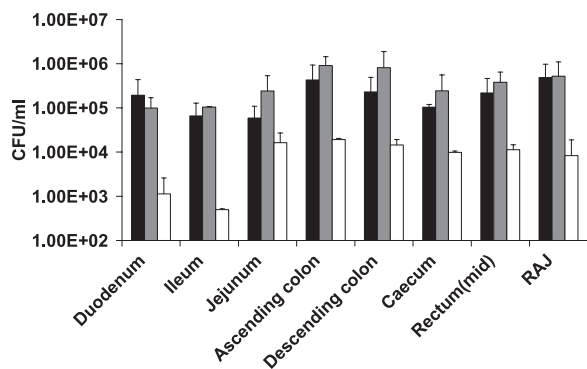


FIG. 4. Adherence of EC355/98 (gray) and NCTC12900 (black) to ovine intestinal tissues. White bars represent resident aerobic flora count (control). There were no significant differences between the results for the tissue sections ($P = 0.053$).

disease were observed. Fecal samples were collected as per the schedule outlined in Materials and Methods, and the numbers of EC335/98 bacteria were detected by direct plating. Excretion was observed for the duration of the experiment (day 36) (Fig. 5), although by this time, only one animal (51088) was excreting detectable EC335/98. At 24 h after oral inoculation, the numbers of EC335/98 bacteria in the feces were in the region of 10^7 CFU g^{-1} feces. Thereafter, the mean number declined quickly and stabilized at day 7 to about 10^4 to 10^5 CFU g^{-1} feces until day 29 (Fig. 5). By this time, not all animals were excreting and detection was sporadic. By day 38, all fecal samples from all animals were negative for EC335/98.

Three lambs that had shed EC355/98 for the longest period prior to ceasing shedding (lambs 51088, 51098, and 51103) were examined postmortem on day 38. EC335/98 bacteria were recovered either by direct count or after enrichment from all lambs (Table 1). One lamb had EC335/98 bacteria detectable

TABLE 1. Recovery of EC335/98 from tissues of lambs 51088, 51103, and 51098 at day 38 p.i.

Tissue	Detection or no. of EC335/98 bacteria (CFU g^{-1}) recovered from indicated lamb ^a		
	51088	51103	51098
Rumen	ND	ND	ND
Duodenum	ND	ND	ND
Jejunum	ND	ND	ND
Ileum samples			
No. 6	3.00E+2	1.00E+2	1.00E+2
No. 5	1.00E+2	ND	ND
No. 4	1.00E+2	+	ND
No. 3	+	+	ND
No. 2	3.00E+2	ND	ND
No. 1	2.00E+2	+	ND
Cecum	+	+	ND
Descending colon	1.00E+2	ND	+
Spiral colon	+	ND	ND
Ascending colon	+	+	ND
Rectum samples			
No. 6	ND	ND	+
No. 5	1.00E+2	+	ND
No. 4	ND	ND	ND
No. 3	+	+	ND
No. 2	+	+	+
No. 1	+	ND	ND
Recto-anal junction	1.00E+2	ND	ND

^a ND, not detected; +, positive for *E. coli* O26 after 6 h enrichment.

by direct counting from many different parts of intestinal tissue, including ileum sections 1, 2, 4, and 6, the descending colon, rectum section 5, and the RAJ. A total of 11 of 18 tissue sections taken from the ileum were positive for EC335/98, but only 1 of 6 ileal sections (ileum 6) was positive for all three lambs.

In a separate experiment, two lambs dosed with EC335/98,

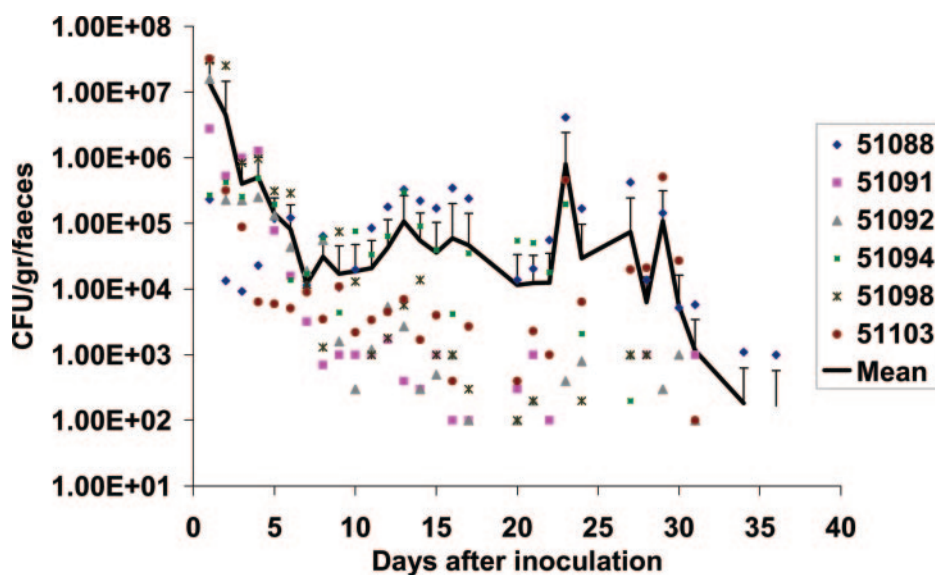


FIG. 5. Recovery of bacteria from the feces of 6-week-old lambs ($n = 6$) inoculated with 1×10^{10} CFU ml^{-1} *E. coli* strain EC335/98. Samples in which no growth was detected after direct plating or enrichment are not shown. Results are presented as the means \pm standard deviations from the means.

TABLE 2. Recovery of EC335/98 from tissues of control lambs 51481 and 51478 at day 4 p.i.

Tissue	No. of EC335/98 bacteria (CFU/g) recovered from indicated lamb	
	51481	51478
Rumen	0.00E+0	7.00E+2
Duodenum	2.70E+3	1.20E+3
Jejunum	8.10E+3	6.00E+2
Ileum samples		
No. 6	6.91E+7	4.48E+4
No. 5	1.43E+9	6.62E+4
No. 4	1.14E+9	1.12E+5
No. 3	9.17E+8	9.43E+4
No. 2	1.00E+6	3.05E+4
No. 1	2.86E+8	4.47E+4
Cecum	1.48E+9	8.41E+4
Descending colon	1.00E+8	6.72E+4
Spiral colon	1.39E+9	6.37E+4
Ascending colon	1.95E+9 ^a	5.36E+4
Rectum samples		
No. 6	2.37E+5	8.85E+4
No. 5	7.46E+7	1.90E+4
No. 4	1.83E+8	5.53E+4
No. 3	7.48E+8	8.20E+4
No. 2	9.46E+7	6.43E+4
No. 1	9.00E+5	1.25E+4
Recto-anal junction	2.20E+7	3.72E+4

^a Small AE lesions were detected, and there were masses of bugs attached to the intestine with lots of luminal bacteria.

as described above, were examined postmortem on day 4. Strain EC335/98 bacteria were recovered by direct counts from all sites tested except for the rumen of one lamb (Table 2). IHC analysis showed that stained *E. coli* O26 bacteria intimately associated with the mucosa of the ascending colon of lamb 51481 and produced small AE lesions (Fig. 6A and B).

Having demonstrated that strain EC335/98 induced lesions in a study of an orally inoculated lamb, we wished to test the other four strains with an alternative in vivo model, the ligated-

TABLE 3. Details of bacteria recovered from feces/tissue and AE lesions in histological sections of ligated spiral colon loops inoculated with 1×10^9 CFU/ml *E. coli* O26 bacteria and the control O157 strain

Loop no.	Strain	Serotype	No. of bacteria recovered from loops (CFU/ml)	IHC ^b result
1	EC417/02	O26:K60	2.46E+10	Small AE lesions
2	EC460/02	O26:K ⁻	1.40E+9	No AE lesions
3	EC740/01	O26:K60	1.20E+11	No classical AE lesions, few bacteria attached
4	EC746/01	O26:K60	8.60E+10	Good AE lesion directly above FAE ^a
5	NCTC12900	O157:H7	2.40E+10	No AE lesions
6	NCTC12900	O157:H7	5.75E+10	No AE lesions

^a Follicle-associated epithelium.

^b Immunohistochemistry.

gut-loop model. The numbers of organisms of each of the four *E. coli* O26 strains tested and control strain NCTC12900 recovered from each tissue sample were similar (Table 3), the differences were not significant ($P = 0.259$), and AE lesions were observed in IHC-stained sections of loops that were inoculated with two of the O26 strains (Table 3). No AE lesions were detected in loops inoculated with the control strain NCTC12900, although lesions have been previously observed in this model with this strain (60) and after oral inoculation in the cecum, colon, rectum, and RAJ of 5-week-old lambs (34); in the distal colon, rectum, and RAJ of 8-week-old goats (32); and in the ileum of 6-day-old goats (62).

DISCUSSION

E. coli serogroup O26 is a public health concern given its prevalence in ruminants (1, 5, 64) and association with human disease (28, 38, 40). Sheep are reservoirs for AEEC O26, and the ovine infection model used in this study may be regarded as appropriate for elucidating the relationship between the host

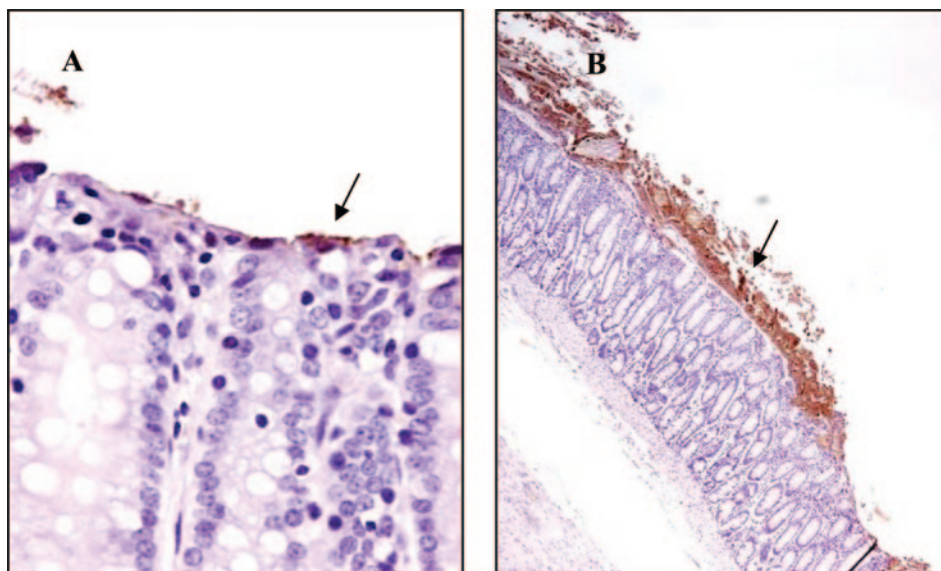


FIG. 6. Ascending colon, lamb 51481, day 4 p.i. IHC showing specifically stained *E. coli* O26 bacteria. (A) Bacteria intimately attached to the mucosa (arrow). Magnification, $\times 400$. (B) Bacteria loosely attached and luminal (arrow). Magnification, $\times 100$.

and this potentially zoonotic pathogen. In vitro tissue culture adherence and invasion assays are recognized as one established method for the investigation of host-pathogen interactions (30). Similarly, IVOC systems are another established method for the study of adhesion and tissue-specific tropisms on gut explants (2, 21, 23, 26). These tools showed that all five O26 strains induced AE lesions on tissue culture as shown by FAS and by TEM, but with no obvious differences in behavior from our well-characterized control strain NCTC12900. Also, comparative studies between strain EC335/98 and strain NCTC12900 of adherence to ovine tissues from distinct sites of the GIT provided no indication of specific or preferential localization of EC335/98. It is possible that the in vitro conditions could have had a negative effect on the expression of genes that favor adherence to a particular site in the gut or that conditions of the experiment were too permissive and adherence was promiscuous. The elegant studies of van Diemen and others (58) strongly suggest that O26 possesses several factors other than the LEE that potentially influence colonization in cattle. Whether these apply to sheep is yet to be tested, and as some of these factors are fimbrial, we have probably not used inducing conditions.

When EC335/98 was orally inoculated into 6-week-old lambs, this strain was shed for beyond a calendar month. There were no obvious differences in overall pattern of excretion between EC335/98 and NCTC12900, which was studied in the same animal model in previous studies (10, 66). After an initial decline in excretion of EC335/98 over the first week, presumably as the bolus passed through, there was a period of sustained excretion of 10^4 to 10^5 CFU g^{-1} feces for a further 20 days or so from most of the animals inoculated. Based on our previous studies of NCTC12900, we found that in this model, in which we demonstrated persistence to be primarily dependent upon intimin (66), it is highly likely that this period of excretion of EC335/98 may also reflect dependence upon intimate attachment. To assess this hypothesis, tissues taken from sacrificed animals on day 4 p.i. were examined histologically, and only small, sparse lesions were observed in the mucosa of the ascending colon but not elsewhere, despite there being very high numbers of O26 bacteria in the samples. Dean-Nystrom et al. have suggested that AE lesion formation by *E. coli* O157 in vivo requires high bacterial cell densities in proximity to susceptible host epithelial cells (15). Whether this applies to O26 requires investigation, but certainly, despite detection of 10^9 CFU associated with many tissues from the GIT, lesions were detected only in the ascending colon and in only 1 of 20 tissues taken. The strain used in vivo produced copious AE lesions on tissue culture and adhered to GIT IVOC tissues efficiently within 6 h. Thus, we conclude there is little correlation between in vitro cell culture and in vivo cell epithelium. Also, it is possible to suggest that strain EC335/98 is able to colonize 6-week-old lambs but that intimate adherence may not be the primary mechanism for persistent colonization. However, care should be taken in drawing this conclusion because previous studies using the model with O157:H7 showed considerable difficulty in detecting AE lesions, and yet, an *eae* mutant was highly attenuated using the same model (66). This requires further analysis.

We also hypothesized that when excretion was no longer detectable by IMS analysis of fecal samples, it was possible that

the animals were still colonized. This is certainly a major risk factor for modeling the epidemiology of transmission. To test this, animals that had shed the longest but were negative by examination of feces at day 38 were sacrificed and their tissues examined. Of the 60 tissue samples examined from these three animals, EC335/98 was recovered from 10 samples by direct plating and a further 18 after enrichment. Here was evidence that this strain could persist longer than normally assessed by fecal analysis. However, no AE lesions were detected when the same tissue samples were examined histologically. It seems likely that lesions, if present at this stage of colonization, may be even sparser than early in infection and exceedingly difficult to detect. Indeed, it may be anticipated that lesions would be resolving as the host recovers from infection. In addition, EC335/98 bacteria were recovered from these three animals from many sites, although the highest numbers were from the ileum. This is in accord with previous data indicating that β -intimin AECC preferentially adhere to the mucosa of the proximal GIT (7, 43). Their presence elsewhere may suggest promiscuity in adherence, possibly intimate adherence, and this needs further study. The presence of detectable lesions in the colon early after oral inoculation followed by the detection of O26 organisms, particularly in the ileum, much later may suggest that a phase of generalized adherence possibly at many sites of the GIT is resolved over time such that the preferred site of adherence, the ileum, remains colonized. It should also be borne in mind that the experiment started with a laboratory-prepared inoculum given orally, probably followed by subsequent inapparent inoculations from the environment via the fecal-oral route. The lack of animals prevented in-contact studies for assessing the impact of these two routes of inoculation, which should be done in future work.

Intestinal-loop models have been used in ruminants to study EPEC and EHEC infections (39, 48, 53) and the immune responses induced by these organisms (22). As we wished to limit the number of animals used in this study, we utilized the ligated-spiral-colon model as a comparative model to assess whether O26 strains inducing AE lesions in vitro tissue culture did so in vivo. Two of the four produced AE lesions; one strain (EC417/01) induced small AE lesions, and the other strain (EC746/01) produced AE lesions that were readily visible directly over the follicle-associated epithelium. Not unsurprisingly, there were differences between strains tested in the same model, as has been noted for *E. coli* O157:H7 in various in vivo models (10, 59). However, it should be borne in mind that serogroup O26 is heterogeneous with those associated with human disease-elaborating Shiga toxins and those commonly found in ruminants lacking Shiga toxins (18, 41, 55). The strains used for this study lacked Shiga toxins and, therefore, may not be representative of those O26 strains associated with human disease. Indeed, without further detailed investigations, which are now in hand, it is not possible to determine whether there exist clonal associations between certain O26 types and certain hosts, although earlier studies suggest close clonality for STEC O26 (50). The strains for this study were selected on the basis of host of origin and the lack of Shiga toxins simply to minimize the complexity of experimentation; in the United Kingdom, STEC bacteria require category level III containment. The variability may reflect innate differences between strains or subtle differences between the local environments

created by the insult. Surprisingly, NCTC12900, which was used as a control in our loop study, did not generate AE lesions in the experiment, whereas previous work showed that it was capable of inducing AE lesions in the spiral colon (60). The cause of this discrepancy is unclear but might be that the immune statuses of the animals used were not equivalent. Although sheep were demonstrated to be free from *E. coli* O157 and O26, it is possible that previous exposure to another AEEC strain may have induced cross-protective immunity. The induction of AE lesions by NCTC12900 has been observed on the intestinal mucosa of 6-week-old lambs and the gastrointestinal tracts of 6-day- and 8-week-old goats after oral inoculation (32, 62).

In conclusion, these experiments indicate that O26 strains lacking Shiga toxins but possessing intimin are capable of intimate adherence in tissue culture models, variably so in ovine ligated-gut loops and the descending colon of one lamb after oral inoculation. One strain was shown to persist for a month after oral inoculation of 6-week-old conventional lambs. The role of intimin in persistence is implicated. Importantly, here is a suitable model for more detailed analysis of interactions between O26 and one of its natural hosts.

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