Colonization of 8-week-old conventionally reared goats by *Escherichia coli* O157 : H7 after oral inoculation


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Enterohaemorrhagic *Escherichia coli* O157 : H7 infections of man have been associated with consumption of unpasteurized goat’s milk and direct contact with kid goats on petting farms, yet little is known about colonization of goats with this organism. To assess the contribution of flagella and intimin of *E. coli* O157 : H7 in colonization of the goat, 8-week-old conventionally reared goats were inoculated orally in separate experiments with \( 1 \times 10^{10} \) c.f.u. of a non-verotoxigenic strain of *E. coli* O157 : H7 (strain NCTC 12900 Nalr), an aflagellate derivative (DMB1) and an intimin-deficient derivative (DMB2). At 24 h after inoculation, the three *E. coli* O157 : H7 strains were shed at approximately \( 5 \times 10^4 \) c.f.u. (g faeces)\(^{-1} \) from all animals. Significantly fewer intimin-deficient bacteria were shed only on days 2 (\( P = 0.003 \)) and 4 (\( P = 0.014 \)), whereas from day 7 to 29 there were no differences. Tissues from three animals inoculated with wild-type *E. coli* O157 : H7 strain NCTC 12900 Nal\(^{+} \) were sampled at 24, 48 and 96 h after inoculation and the organism was cultured from the large intestine of all three animals and from the duodenum and ileum of the animal examined at 96 h. Tissues were examined histologically but attaching-effacing (AE) lesions were not observed at any intestinal site of the animals examined at 24 or 48 h. However, the animal examined at 96 h, which had uniquely shed approximately \( 1 \times 10^7 \) *E. coli* O157 : H7 (g faeces)\(^{-1} \) for the preceding 3 days, showed a heavy, diffuse infection with cryptosporidia and abundant, multifocal AE lesions in the distal colon, rectum and at the recto-anal junction. These AE lesions were confirmed by immunohistochemistry to be associated with *E. coli* O157 : H7.

INTRODUCTION

*Escherichia coli* serotype O157 : H7 infection was first recognized in the early 1980s to be associated with haemorrhagic colitis, haemolytic-uraemic syndrome and thrombocytopenic purpura in man (Karmali et al., 1983; Riley et al., 1983). Human infection and the sequelae of infection have been well documented subsequently (Smith & Scotland, 1993; Boyce et al., 1995; Swinbanks, 1996) and *E. coli* O157 : H7, classified as belonging to the recently defined enterohaemorrhagic *E. coli* pathotype, is regarded worldwide as the leading cause of both haemorrhagic colitis and haemolytic-uraemic syndrome.

Transmission of *E. coli* O157 : H7 is faecal–oral (Pepin et al., 1997; Locking et al., 2001), with cattle considered as the primary reservoir (Griffin & Tauxe, 1991) and sheep also recognized as a significant reservoir (Chapman et al., 1997; Heuvelink et al., 1998; Meng et al., 1998; Pegan & Desmarchelier, 1999). Other potential sources of these organisms have been described, including rabbits and seagulls amongst other species (Griffin & Tauxe, 1991; Pritchard et al., 2001).

Several recent reports cite goats as potential sources of *E. coli* O157 : H7 infection. Shukla et al. (1995) reported four human cases of bloody diarrhoea affecting one adult and three children aged 2–4 years, which were traced to a farm visitor centre in Leicestershire, UK. Strains of *E. coli* O157 : H7 phage type 2, all with identical restriction fragment length polymorphism patterns, were isolated from the cases and from four cattle and six goats. Three other incidents in the UK have implicated goats on visitor farms as potential sources of infection (Chapman et al., 2000; Pritchard et al., 2000; Payne et al., 2003). In continental Europe, Heuvelink...
et al. (2002) showed that two of 11 petting zoos had goats that were positive for *E. coli* O157 : H7. In Bohemia, four cases of haemolytic-uraemic syndrome in children were reported and the source was identified as unpasteurized goat’s milk, with *E. coli* O157 : H7 phage type 2 isolated from a goat and an asymptomatic human carrier who drank goat’s milk (Bielaszewska et al., 1997). An outbreak of *E. coli* O157 : H7 associated with unpasteurized goat’s milk has also been described in Canada (McIntyre et al., 2002). Foschino et al. (2002) reported a 1-7 % incidence rate of *E. coli* O157 : H7 in samples of raw goat’s milk intended for cheese making in the Bergamo region of Italy, although it was not associated with infection in man. Despite these incidents involving the goat as the primary source, the biology of *E. coli* O157 : H7 in this host is not understood. Little is known of the prevalence of *E. coli* O157 : H7 in goats, with only a few reports suggesting low prevalence rates in the region of 0-2 % (Blanco et al., 2003; Dontorou et al., 2004).

Attaching-effacing (AE) *E. coli* belonging to non-O157 serogroups are found frequently in diarrhoeic goats (Duhamel et al., 1992; Drolet et al., 1994) and putative AE *E. coli* have been reported in healthy goats (Cid et al., 2001; de la Fuente et al., 2002; Orden et al., 2003a, b). Furthermore, *E. coli* belonging to serogroup O145 induced AE lesions in an adult goat (Barlow et al., 2004). Collectively, these observations suggest that goats may be a permissive host for AE *E. coli* including *E. coli* O157 : H7. Recently, it was shown that 6-day-old conventional kids were colonized by *E. coli* O157 : H7 and that small AE lesions in the mucosa of the ileum and large intestine were observed following oral inoculation (Wales et al., 2005). These data suggest that goats are susceptible to colonization by *E. coli* O157 : H7, probably in an intimin-dependent manner, but whether this is the case for older goats remains untested. In this study, we wished to assess the contribution of two bacterial factors, intimin and flagella, in colonization. Intimin is considered to play a role as a key effector in the induction of AE lesions, whereas flagella have been shown to contribute to persistence, particularly of commensal *E. coli*, in many species. *E. coli* O157 : H7 strain NCTC 12900 NaI, which has been shown to colonize small ruminants, and isogenic intimin- and flagella-deficient mutants were selected for this study. Weaned, conventional, 8-week-old goats were orally inoculated with the test strains and, to assess colonization, faecal shedding was monitored and serial post-mortem examinations were performed to locate the organism within the gastrointestinal tract.

**METHODS**

**Bacterial strains and inocula.** A derivative of *E. coli* O157 : H7 strain NCTC 12900 that does not possess either stx1 or stx2 verocytotoxin genes (NCTC, Health Protection Agency, Colindale, London, UK) (Best et al., 2005) was made resistant to nalidixic acid at a concentration of 15 µg ml⁻¹ by passage on complex medium supplemented with the antibiotic and designated *E. coli* O157 : H7 strain NCTC 12900 NaI. Mutants defective for the elaboration of flagella and intimin were constructed in *E. coli* O157 : H7 strain NCTC 12900 NaI following the methods described previously by Best et al. (2005). The flagella-deficient derivative was made with a streptomycin-resistance gene cassette inserted in the *fliC* gene, *fliC::strR*, and was designated DMB1. This derivative was non-motile in 0-35 % nutrient agar, did not elaborate flagella as determined by transmission electron microscopy (TEM) and did not agglutinate H7 antisera. The intimin-deficient derivative was made with a chloramphenicol-resistance gene cassette inserted in the *eae* gene, *eae::camR*, and was designated DMB2. This derivative failed to induce AE lesions in HEp-2 cells. The growth rates in Luria–Bertani (LB) broth for both isogenic derivatives were the same as for the parental strain.

Strain NCTC 12900 NaI and isogenic derivatives were stored in heart infusion broth medium supplemented with 30 % (w/v) glycerol on beads at −80 °C and working stocks were stored at room temperature on Dorset’s egg medium.

NCTC 12900 NaI and isogenic derivatives were streaked from Dorset’s egg medium on to sorbitol MacConkey agar (SMAC) plates containing nalidixic acid (15 µg ml⁻¹) and well-isolated colonies were inoculated separately into 100 ml aliquots of LB broth in 250 ml conical flasks. After incubation for 16 h at 37 °C with gentle agitation, the bacterial cells were harvested by centrifugation (3000 g for 10 min) and resuspended in PBS (pH 7-4). The bacterial suspensions contained approximately 1×10⁹ c.f.u. ml⁻¹ as determined by serial dilution and plating.

**Animals.** Post-partum, 20 neonatal goat kids were allowed to suckle and after weaning at about 6 weeks of age were separated from their respective nannies. The animals were selected randomly into three groups comprising eight (A), six (B) and six (C) animals with each group penned separately. Each animal was identified with duplicate ear tags encoding a unique four-digit identification number and housed indoors for a further 2 weeks and provided with standard rations and water *ad libitum.*

Prior to inoculation with *E. coli* O157 : H7, faeces were taken from the rectum of each animal and cultured for *E. coli* O157 : H7, as described below. All animals in each of the three groups were inoculated orally with one challenge strain; group A was challenged with *E. coli* O157 : H7 NCTC 12900 NaI, group B with DMB2 and group C with DMB1. The challenge doses (10 ml) were administered orally using a worming gun, ensuring delivery of the inoculum to the pharynx. Rectal faecal samples were taken from each animal on days 1, 2, 4, 7, 8, 10, 11, 15, 18, 22, 25 and 29 after challenge. Animals were observed at least twice daily to monitor clinical status.

**Necropsy procedures.** Necropsy procedures were as described previously (Wales et al., 2005). Briefly, from group A, goats were selected at random at 24 (animal 1019), 48 (animal 1001) and 96 (animal 1003) h after challenge and tissue samples were collected immediately after euthanasia with barbiturate overdose. Samples were collected from the rumen, duodenum, jejunum, ileum, caecum, ascending colon, spiral colon and from six sites excised at approximately 2 cm apart measuring from the recto-anal junction (RAJ), along the rectum toward the distal colon. Samples were used for bacteriological and histological examination and electron microscopy as described below. Clips and plastic bags were used to enclose the cut ends of the intestinal tract as dissection proceeded, as described previously in sheep (Wales et al., 2001a), to prevent cross-contamination of samples.

**Bacteriological examination.** Faecal samples taken prior to oral inoculation were examined for the presence of *E. coli* O157 following previously described methods (Wales et al., 2001a, b, 2002; Woodward et al., 2003). Faeces (1 g) were resuspended in 9 ml buffered peptone water (BPW), incubated at 37 °C for 6 h and O157 : H7 organisms were recovered by O157-specific immunomagnetic separation and plated on to cefxime-tellurite SMAC plates.
To detect the strains used to inoculate the animals orally, previously described methods were followed (Wales et al., 2001a, b, 2002; Woodward et al., 2003). Faeces (1 g) were resuspended in 9 ml BPW by vortexing and serial dilutions were plated directly on to SMAC plates containing nalidixic acid (15 µg ml⁻¹). Additionally, dilutions were retained overnight at 4 °C and, if there were no direct counts of confirmed O157 organisms, the dilutions were incubated at 37 °C for 6 h and samples plated on to SMAC plates containing nalidixic acid (15 µg ml⁻¹). The serogroup of bacteria recovered by these processes was checked by O157-specific latex agglutination (Oxoid). Tissue samples collected from kids for bacteriological examination were homogenized in BPW (1 g in 9 ml) and processed as described above.

Pathological studies

Light microscopy. Tissues were placed immediately into 10 % neutral buffered formalin at room temperature and left to fix for at least 24 h. Trimmmed tissues were processed routinely to paraffin wax and 4 µm sections were stained with haematoxylin and eosin (H&E) and observed using an Olympus CX41 microscope.

Immunohistochemistry. Tissue blocks were fixed in 10 % neutral buffered formalin, processed to wax and sectioned at 4 µm on a rotary microtome in preparation for immunohistochemistry. Tissue sections were dewaxed in xylene and dehydrated in absolute alcohol before immersion in freshly prepared 3 % hydrogen peroxide/methanol for 10 min to inhibit endogenous peroxidase activity.

Sections were rehydrated in running tap water prior to assembly into the Shandon Sequenza staining system. The slides were washed with 2× sodium chloride/Tris-buffered saline (NaCL/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 Laboratories) for 20 min. E. coli O157-specific polyclonal antibody, raised in rabbits (Veterinary Laboratories Agency, Weybridge, UK), was then applied (diluted 1:1000 and 1:5000 in 2× NaCL/TBS supplemented with 5 % normal rabbit serum) to the sections for 1 h at room temperature (Wales et al., 2001b).

E. coli antigens were visualized following incubation with biotinylated goat anti-rabbit IgG (diluted 1:200, with normal goat serum diluted 1:66 in 2× NaCL/TBS supplemented with 3 % normal sheep serum) for 30 min at room temperature, an avidin–biotin–peroxidase conjugate (Vector Laboratories) for 30 min at room temperature and citrate-buffered diaminobenzidine for 10 min at room temperature. The sections were then counterstained in Meyer’s haematoxylin before being dehydrated in absolute alcohol, cleared in xylene and coverslipped using DPX mountant. The stained tissue sections were then examined by light microscopy.

Electron microscopy. After removal from the animal, the tissues were fixed in 3 % glutaraldehyde prepared in 0·1 M phosphate buffer. Following microscopic examination of the H&E sections, at sites where lesions were identified, corresponding glutaraldehyde-fixed tissues were cut to 1–2 mm thickness for TEM examination. The tissues were then prepared on to copper grids using a diamond knife and stained with uranyl acetate and lead citrate prior to examination using a Phillips CM10 TEM.

Statistical analyses. The sensitivity of detection by direct plating was approximately 500 organisms (g faeces)⁻¹. Samples positive by enrichment were considered to have up to 500 organisms (g faeces)⁻¹ and those samples in which no organisms were detected were given an arbitrary value of 1 to avoid the issue of a zero value giving results to infinity. The non-parametric Kruskal–Wallis test was used to compare the mean group counts for each occasion. When this was significant at P ≤ 0·05, the individual group means were compared by a multiple comparison test. The percentages of animals positive were also compared by Fisher’s exact test at each time point.

RESULTS

Clinical findings

All animals in all study groups remained clinically normal throughout the experiment, with no evidence of pyrexia or diarrhoea. Gross pathological changes were not observed at necropsy in the three animals examined at 24, 48 and 96 h after oral inoculation.

Faecal shedding of E. coli O157 : H7

All faeces samples collected from the animals prior to the experimental procedure were negative for E. coli O157 as assessed by immunomagnetic separation. The oral inoculation doses were determined to be 1 × 10¹⁰ c.f.u. for each kid in each of the three groups.

The faecal shedding data are shown in Fig. 1. At 24 h after challenge, all animals in all groups were shedding the inoculated strain [range 2 × 10⁴–9·2 × 10⁶ c.f.u. (g faeces)⁻¹], with no statistically significant differences between the geometric mean values of the three groups at this time.

On days 2 and 4 after inoculation, differences were observed. On these days, all animals in groups A and C dosed with E. coli O157 : H7 strain NCTC 12900 Nal⁻ and DMB1 (isogenic aflagellate derivative), respectively, shed the inoculated strain at about the same numbers as on day 1, whereas DMB2 (isogenic intimin-deficient derivative) was detected in four and three of the six animals in group B on days 2 and 4 after challenge, respectively. The only statistically significant difference in numbers of animals shedding was at day 4 (P = 0·021). On days 2 and 4, the geometric mean shedding scores in groups A and C [~5 × 10⁴ c.f.u. (g faeces)⁻¹] were significantly higher than the geometric mean shedding score for group B animals [~5 × 10¹ and 1 × 10² c.f.u. (g faeces)⁻¹; day 2, P = 0·003; day 4, P = 0·014]. The geometric mean counts from group B animals were lower than those from the other two groups on days 7 and 8, but these differences were not significant (P = 0·057 and P = 0·062, respectively).

From day 10 onwards, only a few animals shed O157 organisms in each group and there were no statistically significant differences between groups or strains. However, it was noted that E. coli O157 : H7 strain NCTC 12900 Nal⁻ was not detected in faecal samples tested on days 22, 25 and 29, whereas both DMB1 and DMB2 were detected, albeit in small numbers, on these days.
Bacteriological findings at necropsy

Three animals in group A, inoculated with *E. coli* O157:H7 strain NCTC 12900 Naïve, were selected at random for post-mortem examination on days 1 (animal 1019), 2 (animal 1001) and 4 (animal 1003). On the day of post-mortem examination, faeces were taken and examined for *E. coli* O157:H7. Animal 1019 had $2 \times 10^4$ c.f.u. (g faeces)$^{-1}$ and animal 1001 had $2 \times 10^4$ c.f.u. (g faeces)$^{-1}$ on day 1 and $7 \times 10^5$ c.f.u. (g faeces)$^{-1}$ on day 2. By contrast, animal 1003 had $9.2 \times 10^6$ c.f.u. (g faeces)$^{-1}$ on day 1, $1.7 \times 10^7$ c.f.u. (g faeces)$^{-1}$ on day 2 and $3.5 \times 10^7$ c.f.u. (g faeces)$^{-1}$ on day 4.

There were differences in the numbers of *E. coli* O157:H7 strain NCTC 12900 Naïve recovered from tissues in individual animals (Fig. 2). *E. coli* O157:H7 was not detected in the small intestine of animal 1019. However, *E. coli* O157:H7 was detected in the jejunum of animal 1001 and the duodenum, jejunum and ileum of animal 1003. In the large intestine, by contrast, *E. coli* O157:H7 was detected in most sites in all three animals. In the case of animal 1003, large numbers of *E. coli* O157:H7 were found at all intestinal sites examined, with up to $10^6$ c.f.u. *E. coli* O157:H7 (g tissue)$^{-1}$ in the mid-section of the rectum. The rumen was negative for all three animals.

![Fig. 1. Persistence of *E. coli* O157:H7 NCTC 12900 Naïve and isogenic flagella (DMB1) and intimin (DMB2) mutants as assessed by faecal shedding.](image)

![Fig. 2. Counts of *E. coli* O157:H7 NCTC 12900 Naïve from tissues collected post-mortem from the gastrointestinal tract of conventional 8-week-old goats at 1 (open bars), 2 (shaded bars) and 4 (filled bars) days post-inoculation with *E. coli* O157:H7. A. colon, Ascending colon; S. colon, spiral colon.](image)
**Histopathological findings**

Significant changes were confined to the distal colon, rectum and RAJ of animal 1003, examined on day 4. The most prominent feature was the presence of large numbers of cryptosporidium organisms that were identified attached to the mucosa, along with scattered, focal, AE lesions associated with adherent bacteria (Fig. 3a). Cryptosporidia and associated bacteria appeared to be co-located on the mucosal surface. Associated with these lesions, the lamina propria was infiltrated with moderate numbers of mixed lymphoid cells and polymorphonuclear leukocytes. The remaining large intestine samples (ascending colon and spiral colon) were negative for cryptosporidia and attached bacteria.

Sections of small intestine examined were less well preserved than the large intestine. The epithelium in the majority of samples was still intact, although some artefactual separation of the underlying lamina propria had occurred. Small numbers of coccidia were identified in the ileum, predominantly in the crypt epithelium of animal 1001, examined post-mortem at day 2. Cryptosporidia and attached bacteria were not identified. Abnormalities were not detected in the rumen.

Attempts to isolate cryptosporidia from animal 1003 could not be made as the samples had been disposed of by the time the results from the histological examination were known.

**Immunohistochemistry**

Multifocal, variable-sized colonies of *E. coli* O157:H7 organisms were identified at all sites of the distal colon, rectum and RAJ in animal 1003 (Fig. 3b). These tended to cover from one or two to several adjacent epithelial cells. In addition, lesions identified in the mucosa at the RAJ were found on both lymphoid-associated epithelium and non-lymphoid-associated epithelium.

**TEM**

Cryptosporidia and AE lesions associated with bacteria were confirmed on the intestinal mucosa (Fig. 4). Both organisms were frequently co-located on the same cell (Fig. 4b), confirming the light microscopic findings.

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**Fig. 3.** Microscopic sections of rectum from goat 1003 taken 4 days post-inoculation with *E. coli* O157:H7. (a) Cryptosporidia and AE *E. coli* (arrows) are present on the mucosa. H&E staining, magnification ×400. (b) Focal AE *E. coli* immunolabelled by the *E. coli* O157 antiserum are present on the epithelial surface (arrows). Cryptosporidia remain unstained (arrowhead). Immunoperoxidase staining, magnification ×400.

**Fig. 4.** Transmission electron microscopic sections of rectum from goat 1003 taken 4 days post-inoculation with *E. coli* O157:H7. (a) Bacteria intimately attached to the surface of an enterocyte with pedestal formations and effacement of microvilli. Magnification ×20 000. (b) E, AE *E. coli* attached to the surface of two epithelial cells; C, a cryptosporidium organism co-located on one of the cells. Magnification ×10 000.
DISCUSSION

It is established that enterohemorrhagic \textit{E. coli} O157 : H7 colonizes the intestinal tract of cattle and sheep, often leading to long-term persistent infection (Cray & Moon, 1995; Brown et al., 1997; Dean-Nystrom et al., 1999), and that intimin has been identified as a significant bacterial factor in the colonization of these species (Cornick et al., 2002; Woodward et al., 2003). In this study, faecal shedding was used as an indicator of the extent of intestinal colonization. Twenty-four hours after oral inoculation, the number of organisms, approximately $10^4$–$10^5$ c.f.u. (g faeces)$^{-1}$, was equivalent for each of the test strains. Strain NCTC 12900 Nal$^\text{R}$ and the flagella-deficient mutant continued to be shed in faeces at about this level by all animals for the following week, whereas shedding of the intimin-deficient mutant from day 2 onwards was from fewer animals and with smaller numbers of organisms detected. Whilst these data suggested that intimin might play a role in intestinal colonization in the weaned goat, statistically significant differences in shedding of the intimin-deficient mutant were only noted on days 2 and 4 after inoculation, with no significant differences observed between the three test strains thereafter. Indeed, strain NCTC 12900 Nal$^\text{R}$ was eliminated by 3 weeks, whereas shedding of the intimin-deficient mutant from day 2 onwards was from fewer animals and with smaller numbers of organisms detected. Without further experimentation aimed at addressing this, it is not possible to draw any firm conclusions.

Recent studies in cattle reported by Naylor et al. (2003) suggested a preferred site of colonization and AE lesion formation by \textit{E. coli} O157 : H7 at the RAJ. Sheng et al. (2004) confirmed these findings in cattle but did not cite any data on colonization of sheep following rectal administration of the inoculum. In the studies reported here, the RAJ and six other sites along the rectum to the distal colon of the goats were examined. Lesions were not detected in this region of the animals examined 1 and 2 days after oral inoculation but were present in the animal examined on day 4 (animal 1003). This animal shed large numbers of \textit{E. coli} O157 : H7, in the region of $10^6$–$10^7$ c.f.u. (g faeces)$^{-1}$, and had approaching $10^6$ organisms (g tissue)$^{-1}$ in the mid-rectum. This high density of \textit{E. coli} O157 : H7 may have been sufficient to induce the AE lesions. However, the AE lesions were not associated specifically with lymphoid tissue in the RAJ, as has been described for cattle (Naylor et al., 2003). The close association of AE lesions with cryptosporidia is an interesting observation.

In natural and experimental infections in goats, \textit{Cryptosporidium parvum} causes severe clinical disease, often with high morbidity and mortality (Koudela & Bokova, 1997; Johnson et al., 1999), and severe lesions are induced, often in the posterior jejunum and ileum (Koudela & Jiri, 1997). However, asymptomatic carriage of cryptosporidia has been described in adult goats (Noordeen et al., 2002). In the study reported here, the affected goat was asymptomatic and yet had cryptosporidia associated with the mucosa of the distal colon, rectum and RAJ, a region of the intestine at which cryptosporidia have been observed in the later stages of infection in calves (Tzipori et al., 1983). The observation of concurrent infections with two or more enteropathogens, including cryptosporidia in association with \textit{E. coli}, has been made previously in diarrhoeic calves (Moon et al., 1978; Janke et al., 1990; de la Fuente et al., 1999; Gunning et al., 2001). Based on these recorded concurrent infections and data from the experiments reported in this study, the question arises as to the possible significance of the presence of cryptosporidium infection upon colonization and shedding of \textit{E. coli} O157 : H7. It is possible that either the cryptosporidia were incidental or that prior infection with cryptosporidia may predispose the host to colonization by AE \textit{E. coli}. Without further experimentation aimed at addressing this, it is not possible to draw any firm conclusions.

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