Influence of colostrum deprivation and concurrent *Cryptosporidium parvum* infection on the colonization and persistence of *Escherichia coli* O157:H7 in young lambs

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Escherichia coli O157:H7 and *Cryptosporidium parvum* infections of man have been associated with direct contact with small ruminants. Colostrum protects neonates against gastrointestinal pathogens, and orphan lambs, which are common on petting farms, may be deprived of this protection. In a recent study, it was demonstrated that high shedding of *E. coli* O157:H7 by an 8-week-old goat kid was associated with coincidental *C. parvum* infection. Furthermore, both pathogens were co-located in the distal gastrointestinal tract. It was hypothesized that colostrum deprivation and pre-infection with *C. parvum* predisposed young ruminants to colonization and increased shedding of *E. coli* O157:H7. To test this, 21 lambs 5 weeks of age were divided into four groups as follows: (A) colostrum-deprived and inoculated with *E. coli* O157:H7, (B) colostrum-deprived and inoculated with *C. parvum* and then *E. coli* O157:H7, (C) conventionally reared and inoculated with *E. coli* O157:H7, (D) conventionally reared and inoculated with *C. parvum* and then *E. coli* O157:H7. *C. parvum* was detected between 8 and 12 days post-inoculation in most of the infected lambs. At 24 h post-inoculation with *E. coli* O157:H7, all lambs were shedding between 5×10^4 and 5×10^7 c.f.u. *E. coli* O157:H7 per gram of faeces. *E. coli* O157:H7 was shed in higher numbers in the groups pre-inoculated with *C. parvum*, whether conventionally reared or colostrum-deprived. Interestingly, for the colostrum-deprived lambs on day 3, a significant difference in shedding of *E. coli* O157:H7 was observed ($P=0.038$), with the lambs inoculated with *E. coli* alone yielding higher counts than those pre-inoculated with *C. parvum*. From day 15 onwards, shedding of *E. coli* O157:H7 was highest from the colostrum-deprived *C. parvum*-infected lambs, then (in descending order of shedding) the colostrum-deprived lambs, the conventionally reared lambs infected with *C. parvum*, and the conventionally reared animals. In total, four animals were euthanized, two at 24 h and two at 96 h post inoculation with *E. coli* O157:H7 (two conventionally reared and two colostrum-deprived). All animals euthanized were from groups pre-inoculated with *C. parvum* prior to challenge with *E. coli* O157:H7. On examination of tissues, in three of the four animals examined, multifocal attaching and effacing lesions were observed in the caecum, colon, rectum and at the recto-anal junction, and were confirmed by immunohistochemistry to be associated with *E. coli* O157:H7.

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Abbreviations: AE, attaching and effacing; GIT, gastrointestinal tract; HE, haematoxylin and eosin; IFAT, indirect fluorescent antibody test; IMS, immunomagnetic separation; mZN, modified Ziehl–Neelsen; p.i., post-infection; RAJ, recto-anal junction; VLA, Veterinary Laboratories Agency.

INTRODUCTION

Two pathogens whose incidence in human disease has increased significantly over the last decade are *Escherichia coli* O157:H7 (Kaper *et al.*, 2004) and *Cryptosporidium parvum* (Guerrant, 1997). Both pathogens are of zoonotic

importance and have potential economic implications worldwide.

E. coli serotype O157:H7 infection has emerged as an important cause of human disease in the developed world. It was first recognized in the early 1980s to be associated with haemorrhagic colitis (HC), haemolytic-uraemic syndrome (HUS) and thrombocytopenic purpura (TCP) in man (Karmali *et al.*, 1983; Riley *et al.*, 1983). Human infection has been well documented (Smith & Scotland, 1993; Boyce *et al.*, 1995; Swinbanks, 1996), and *E. coli* O157:H7, classified as belonging to the enterohaemorrhagic *E. coli* (EHEC) pathotype, is regarded worldwide as the leading cause of both HC and HUS (Paton & Paton, 1998).

Transmission of *E. coli* O157:H7 is faecal-oral (Pepin *et al.*, 1997; Locking *et al.*, 2001), with cattle considered to be the primary reservoir (Hancock *et al.*, 1997; Zhao *et al.*, 1995; Griffin & Tauxe, 1991), although sheep and goats are also recognized as significant reservoirs (Chapman *et al.*, 1997; Heuvelink *et al.*, 1998, 2002; Meng *et al.*, 1998; Fegan & Desmarchelier, 1999). The transfer of *E. coli* O157:H7 among adult animals has been the subject of much research, with few firm conclusions; however, for young animals, colostrum deprivation is a risk factor for increased susceptibility to gastrointestinal pathogens (Kelleher & Lonnerdal, 2001), and specifically *E. coli* O157:H7 in cattle (Dean-Nystrom *et al.*, 1997; Rugsbjerg *et al.*, 2003), pigs (Dean-Nystrom *et al.*, 2002) and goats (La Ragione *et al.*, 2005b). Indeed, orphan lambs which are deprived of colostrum and bottle fed with milk replacer on petting farms are a known source of human infection (Chapman *et al.*, 2000; Pritchard *et al.*, 2000; Payne *et al.*, 2003).

Intestinal cryptosporidiosis is caused by *C. parvum*, a widely distributed protozoan parasite, which infects wild animals and many domesticated ones, as well as humans, chiefly immunocompromised individuals (O'Donoghue, 1995; Griffiths, 1998). Since its first description in cattle (Panciera *et al.*, 1971; Barker & Carbonell, 1974), *C. parvum* has been attributed an increasingly important role in neonatal diarrhoea syndrome in domestic livestock. A study by Munoz-Fernandez *et al.* (1996) showed *C. parvum* to be the most frequent aetiological agent involved in outbreaks of diarrhoea in lambs in Europe. Cryptosporidiosis is most common in young lambs (Foreyt, 1990), and may result in poor feed to weight conversion and occasionally death. During the incubation period and clinical course of the disease, the parasite proliferates mainly in the jejunum and the ileum. However, after the start of oocyst shedding, the lesions can spread to other parts of the small and the large intestine (de Graff *et al.*, 1999).

C. parvum has been shown to be a common agent associated with diarrhoeal disease in young calves in Europe, and in a study by De la Fuente *et al.* (1999), mixed infections with other enteric pathogens were reported in approximately 50% of cases of diarrhoea. Although mixed infections are more commonly detected in clinically affected animals

(Morin *et al.*, 1980; Reynolds *et al.*, 1986), the significance of concurrent infections is unclear, particularly with regard to the effect on colonization and shedding of *E. coli* O157:H7. Recently, an 8-week-old goat kid experimentally inoculated with *E. coli* O157:H7 shed high levels of the organism [10^7 c.f.u.(g faeces) $^{-1}$] and was found at post-mortem examination to have a concurrent *C. parvum* infection (La Ragione *et al.*, 2005a). The *E. coli* O157:H7 attaching and effacing (AE) lesions and cryptosporidia were co-located on the mucosa of the large intestine. Also, a high incidence of *C. parvum* in orphan lambs in the UK has been reported, so that especially on petting farms, the risk to human health is increased (G. C. Pritchard, personal communication).

Given that neonates and young animals are protected from most gastrointestinal infections when sucking (Altmann & Mukkur, 1983), we wished to assess the longer-term impact of deprivation of colostrum and ewes' milk upon colonization of lambs beyond weaning age with *E. coli* O157:H7. Additionally, we wished to assess the potential contribution of pre-infection with *C. parvum* in the lamb model used in these studies.

METHODS

Bacteria, parasites and inocula. A derivative of *E. coli* O157:H7 strain NCTC 12900 that does not possess either *stx1* or *stx2* verocytotoxin genes, but is *eae* positive (National Collection of Type Cultures, Health Protection Agency, Colindale, UK), was made resistant to nalidixic acid (Sigma) at $15 \mu\text{g ml}^{-1}$ by passage on complex medium supplemented with the antibiotic, and designated *E. coli* O157:H7 strain NCTC 12900 Nal^r (Best *et al.*, 2005; La Ragione *et al.*, 2005a).

Strain NCTC 12900 Nal^r was stored in heart infusion broth (HIB; Oxoid) 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 Nal^r was streaked from Dorset's egg medium onto SMAC plates (Oxoid) containing $15 \mu\text{g}$ nalidixic acid ml^{-1} , and well-isolated colonies were inoculated separately into 100 ml aliquots of LB broth (Oxoid) 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 (0.1 M, pH 7.4). The bacterial suspensions contained approximately 1×10^9 c.f.u. ml^{-1} , as determined by serial dilution and triplicate plating on SMAC plates.

The *C. parvum* isolate used in these studies was from a field case of diarrhoea in a calf, confirmed by standard Veterinary Laboratories Agency (VLA) testing protocols to have been associated with *C. parvum*. The inocula employed to dose lambs were made directly from the faeces of this calf by resuspension in PBS (0.1 M, pH 7.4) and centrifugation to remove faecal debris. Oocysts were suspended in PBS at 1×10^6 oocysts ml^{-1} and stored at 4°C until required.

Animals. Nine cross-bred lambs from multiple births (leaving at least one lamb with its mother) were separated from their mothers immediately after birth and placed into two groups of three and six animals, respectively (groups A and B). These animals were bottle fed with milk replacer. A further 12 neonatal lambs were allowed to suckle, and after weaning at approximately 4 weeks of age, were separated from their mothers and placed in two groups (C and D)

of five and seven animals, respectively. Each animal was identified with duplicate ear tags encoding a unique four-digit identification number. The animals were housed indoors in groups which allowed oral-faecal contact, and were provided with standard rations and water *ad libitum*. At 4 weeks of age and prior to inoculation with *E. coli* O157:H7 or *C. parvum*, faeces were taken *per rectum* from each animal and cultured for *E. coli* O157:H7. The same samples were tested for cryptosporidia by a modified Ziehl–Neelsen (mZN) method and by the indirect fluorescent antibody test (IFAT). At 5 weeks of age, the lambs in groups B and D were dosed orally with 5×10^5 and 1×10^6 cryptosporidia oocysts, respectively. At 6 weeks of age all groups (A–D) were dosed orally with 1×10^{10} c.f.u. *E. coli* O157:H7. All inocula were delivered in a 10 ml volume using a worming gun (Novartis Animal Health) ensuring that the whole inoculum was delivered directly to the pharynx. Faecal samples were taken *per rectum* by digital insertion. Lambs euthanized for necropsy and at the end of the study (day 39) were euthanized by intravenous barbiturate overdose. All procedures were conducted under the jurisdiction of Home Office licence 70/5441 granted under the Animals (Scientific Procedures) Act (1986).

Preparation and necropsy procedures for animals studied post-mortem. Two colostrum-deprived lambs [tagged 1458 (lamb 1) and 1470 (lamb 2), both group B] and two conventionally reared ('conventional') lambs [tagged 1486 (lamb 3) and 1489 (lamb 4), both group D] that had been challenged with both *C. parvum* and *E. coli* O157:H7 were euthanized and necropsied as described previously (Wales *et al.*, 2001a, b). Briefly, one lamb from each group was examined 24 h (1 and 3) and 96 h (2 and 4) after challenge with *E. coli* O157:H7. These animals were euthanized with a barbiturate overdose, and tissue samples were collected immediately thereafter. Tissue samples were collected from the rumen, duodenum, jejunum, ileum, caecum, ascending colon, spiral colon and recto-anal junction (RAJ), and from six sites excised at approximately 2 cm intervals measured from the RAJ along the rectum toward the distal colon, as described previously (Wales *et al.*, 2001a).

Bacteriological examination. Faecal samples taken prior to oral inoculation were examined for the presence of *E. coli* O157:H7 by previously described methods (Wales *et al.*, 2001a, b, 2005; Woodward *et al.*, 2003). Briefly, faeces (1 g) was resuspended in 9 ml buffered peptone water (BPW; Oxoid), incubated at 37 °C for 6 h statically, and *E. coli* O157:H7 organisms were recovered by O157-specific immunomagnetic separation (IMS; Dynal) and plated onto CT-SMAC plates (Oxoid).

To detect the inoculated strains, previously described methods were followed (Wales *et al.*, 2001a, b; 2005; Woodward *et al.*, 2003; La Ragione *et al.*, 2005a). Briefly, faeces (1 g) was resuspended in 9 ml BPW by vortexing, and serial dilutions were plated directly onto SMAC plates containing 15 µg nalidixic acid ml⁻¹. Additionally, dilutions were retained overnight at 4 °C and, if there were no direct counts of confirmed *E. coli* O157:H7, the highest dilution was incubated at 37 °C for 6 h and samples were then plated on SMAC plates containing 15 µg nalidixic acid ml⁻¹. The serogroup of bacteria recovered by these processes was verified by *E. coli* O157-specific latex agglutination (Oxoid). Tissue samples were processed as described above.

Parasitological studies. Tissues and faeces were examined for the presence of cryptosporidia by both the mZN method and the IFAT. For the mZN stain, the methods were essentially as described previously (Henriksen & Pohlenz, 1981). Briefly, a smear of fresh faeces or a deep tissue scrape was prepared on a microscope slide. Fixed slides were then immersed in cold carbol fuchsin, rinsed in water, decolorized in 5% sulphuric acid, and rinsed in water. The preparation was then counter-stained in 5% malachite green, rinsed in tap water, air-dried, and examined at a magnification of $\times 400$. For

the IFAT, a fresh faecal sample or deep tissue scrape was smeared within the well of a 4-well microscope slide, fixed by carefully applying 50 µl methanol to each well, and then allowed to air dry. Once dry, 30 µl anti-*Cryptosporidium* immunofluorescent antibody (IFA) (TCS Water Biosciences) was applied, and the slide placed in a humidified staining chamber and incubated for 45 min at 37 °C. Excess IFA stain was carefully aspirated from each well and replaced with distilled water for approximately 1 min. Excess water was aspirated and the smear air-dried. Once dry, 20 µl UV-compatible mounting fluid was added and a cover slip placed over the preparation. The cover slip was then sealed with DPX mountant. Preparations were examined using a fluorescence microscope fitted with a 450–490 nm excitation filter and 20 $\times$ or 40 $\times$ objectives. *C. parvum* was identified as apple-green epifluorescent, slightly ovoid to spherical oocysts, 4–6 µm in diameter.

Pathological studies

Light microscopy. At necropsy, tissues were placed immediately into 10% neutral buffered formalin at room temperature and left to fix for at least 24 h. Trimmed tissues were processed routinely to paraffin wax. Sections were cut at 4 µm and stained with haematoxylin and eosin (HE). Selected tissues were also stained using the Giemsa method.

Immunohistochemistry. Essentially, methods were as described previously (La Ragione *et al.*, 2005a; Wales *et al.*, 2001b). Briefly, tissue blocks were fixed in 10% neutral buffered formalin, processed to wax and sectioned at 4 µm. Sections were rehydrated prior to assembly into the Shandon Sequenza staining system. Slides were washed with 2 $\times$ sodium chloride Tris-buffered saline [0.005 M Tris-buffered saline (TBS), pH 7.6, 1.7% NaCl] before incubation at room temperature with normal goat serum (Vector Laboratories). *E. coli* O157:H7-specific polyclonal antibody (VLA, Weybridge), was then applied (1:1000 and 1:5000 diluted in 2 $\times$ NaCl TBS supplemented with 5% normal rabbit serum). *E. coli* antigens were visualized following incubation with biotinylated goat anti-rabbit IgG. Sections were counter-stained in Meyer's haematoxylin.

Transmission electron microscopy. Essentially, methods were as described previously (La Ragione *et al.*, 2005a). Briefly, glutaraldehyde-fixed tissues were cut to 1–2 mm in thickness. Tissues were then washed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated by immersion in a series of alcohol solutions increasing to 100% alcohol, and placed in propylene oxide prior to embedding in araldite resin. The resin was polymerized at 60 °C for 48 h. Ultrathin sections at 70–90 nm thickness were prepared on copper grids and stained with uranyl acetate and lead citrate.

Confocal microscopy. After de-waxing and rehydration, tissues were placed in PBS prior to preparation for examination by confocal microscopy. Briefly, sections were permeabilized in PBS containing 0.1% Triton X-100, followed by detection of *E. coli* using a FITC-labelled affinity-purified antibody to *E. coli* O157:H7 produced in goats. The sections were then washed thoroughly using PBS and mounted in Vectashield containing 4,6-diaminidino-2-phenylindole (DAPI; Vector Laboratories). Images of the FITC-labelled *E. coli* were obtained by confocal laser scanning microscopy using a Leica TCS SP2 AOBs confocal system attached to a Leica DM IRE2 microscope equipped with Ar-Kr laser excitation (488 nm) and a diode laser (405 nm). An oil-immersion objective lens ($\times 63$, numerical aperture 1.32) was used, and imaging parameters were selected to optimize resolution.

Statistical analyses. The sensitivity of detection by direct plating was approximately 500 c.f.u.(g faeces)⁻¹. Samples positive by enrichment were considered to have up to 500 c.f.u.(g faeces)⁻¹, and those samples in which no organisms were detected were given an

arbitrary value of 1 to avoid the use of a zero value giving results to infinity. *t* tests were used to compare the mean counts transformed to $\log_{10}(\text{count} + 1)$ 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. However, a change in faecal consistency was observed for the animals in groups B and D challenged with *C. parvum* between 5 and 8 days after oral dosing, whereby the faeces became smooth and sticky in consistency (*E. coli* O157:H7 inoculated at day 7).

Faecal shedding of *C. parvum*

Prior to experimental inoculation with *C. parvum*, all animals were confirmed to be free from *C. parvum* by analysis of faeces by mZN and IFAT. In both the conventional and

colostrum-deprived lambs, *C. parvum* was detected in the faeces 24 h after inoculation in all animals except 1493 (group D), which remained negative throughout the study. In addition, *C. parvum* was only detected on day 1 post-inoculation in lamb 1487 (group D). In all other lambs, the organism persisted for between 8 and 12 days, with the exceptions of lambs 1492 and 1496 (both group D), in which *C. parvum* was detected in the faeces 39 days post-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:H7, as assessed by IMS.

The individual animal faecal-shedding data are shown in Fig. 1. At 24 h after oral inoculation, only two animals (1487 and 1492, both group D) were not shedding *E. coli* O157:H7, although by 72 h after oral inoculation, all

Fig. 1. Persistence of *E. coli* O157:H7 NCTC12900 NaI^r as assessed by faecal shedding in 6-week-old lambs. (A) Colostrum-deprived and inoculated with *E. coli* O157:H7, (B) colostrum-deprived and inoculated with *C. parvum* and then *E. coli* O157:H7, (C) conventional and inoculated with *E. coli* O157:H7, (D) conventional and inoculated with *C. parvum* and then *E. coli* O157:H7.

animals in all groups were shedding in the range 10^3 – 10^8 c.f.u.(g faeces)⁻¹.

For the colostrum-deprived lambs (groups A and B) on day 3, a significant difference in shedding was observed ($P=0.038$) in the counts of *E. coli* O157:H7, whereby the counts from the lambs inoculated with *E. coli* O157:H7 alone were higher than those from lambs pre-inoculated with *C. parvum*. Thereafter, the *E. coli* O157:H7 counts were consistently lower for the lambs inoculated with *E. coli* O157:H7 alone compared to those for lambs pre-inoculated with *C. parvum*.

For the conventional lambs (groups C and D) on day 3, a significant difference in shedding ($P=0.019$) was observed, with lambs inoculated with *E. coli* O157:H7 alone having higher counts than those pre-inoculated with *C. parvum*. Thereafter, the *E. coli* O157:H7 counts were consistently lower for the lambs inoculated with *E. coli* O157:H7 alone compared to those from lambs pre-inoculated with *C. parvum*.

From day 15 onward, the numbers of animals shedding in each group were low, and the statistical analysis indicated that the differences between the groups were not significant. However, the shedding frequency at and beyond day 15

(expressed as the number of positive faecal samples over the number of faecal samples examined per test group) was 0.16 for group C (conventional), 0.33 for group D (conventional plus *C. parvum*), 0.46 for group A (colostrum-deprived) and 0.625 for group B (colostrum-deprived plus *C. parvum*).

Post-mortem findings

Bacteriological and parasitological findings. There were differences in the numbers of *E. coli* O157:H7 strain NCTC 12900 Nal^r recovered from tissues in individual animals (Fig. 2). *E. coli* O157:H7 was not detected in the duodenum of any animal examined, or the jejunum of animals 1 (group B), 3 and 4 (group D). *E. coli* O157:H7 was only recovered from the ileum and rumen of animals 2 and 3. By contrast, in the large intestine, *E. coli* O157:H7 was detected at all sites examined in all four animals, and in high numbers [5×10^6 c.f.u. (g tissue)⁻¹] in tissues from the rectum and spiral colon of animal 2 (group B). The *E. coli* O157:H7 counts for the caecum of animal 1 (group B) were particularly high [1×10^7 c.f.u. (g tissue)⁻¹] and numerous AE lesions were observed in these tissues (see below). The overall numbers of *E. coli* O157:H7 recovered from the GIT were notably higher (by

Fig. 2. Counts of *E. coli* O157:H7 NCTC 12900 Nal^r from tissues collected post-mortem from the GIT of lambs 1 and 3 (day 1) and lambs 2 and 4 (day 4) after oral inoculation with *E. coli* O157:H7. A. colon, ascending colon. S. colon, spiral colon.

approximately two orders of magnitude) in the colostrum-deprived lambs compared to the conventional lambs.

In lamb 1 (colostrum-deprived plus *C. parvum*, group B), necropsied 24 h after *E. coli* O157:H7 inoculation, cryptosporidia were detected in the rumen, duodenum, jejunum, ileum, caecum, ascending colon, spiral colon and proximal rectum. Cryptosporidia were found in the rumen, duodenum and spiral colon of lamb 3 (conventional plus *C. parvum*, group D) necropsied 24 h after *E. coli* infection. Cryptosporidia were not detected in tissues collected at necropsy from animals 2 (colostrum-deprived plus *C. parvum*, group B) and 4 (conventional plus *C. parvum*, group D) necropsied 96 h after *E. coli* infection.

Gross and histopathological findings. Gross pathological changes were not observed post-mortem in any of the four animals examined. On histopathological examination, significant changes were confined to the caecum, rectum and RAJ of animals 1 (colostrum-deprived plus *C. parvum*, 24 h, group B), 2 [colostrum-deprived plus *C. parvum*, 96 h post-infection (p.i.), group B] and 4 (conventional plus *C. parvum*, 96 h p.i., group D). Most of the mucosa at all sites appeared normal. However, where scattered foci of closely adherent bacteria were seen in the caecum, rectum (Fig. 3) and RAJ, some epithelial cells associated with bacteria had rounded up. AE lesions were not identified in animal 3 (conventional plus *C. parvum*, group D, examined 24 h p.i.); however, *E. coli* O157:H7 cells were identified by immunohistochemistry (IHC) in the lumen of the caecum and rectum of this animal. Cryptosporidia were not observed on the mucosal surface in any animal.

Sections of small intestine (duodenum, jejunum and ileum) were less well preserved than the large intestine, and some artefactual separation of the epithelium had occurred. *C.*

Fig. 3. Lamb 4 (conventional plus *C. parvum*, group D) 4 days p.i. with *E. coli* O157:H7, rectum. AE *E. coli* (arrows) are present on the mucosa. HE staining; bar, 50 µm. Inset: focal AE *E. coli* immunolabelled by *E. coli* O157 antiserum are present on the epithelial surface (arrow). Immunoperoxidase staining; bar, 50 µm.

Fig. 4. Lamb 4 (conventional plus *C. parvum*, group D) 4 days p.i. with *E. coli* O157:H7, rectum. Focal AE *E. coli* immunolabelled by *E. coli* O157:H7 antiserum are present on the epithelial surface (green). Bar, 20 µm.

parvum was only identified in the ileum of animal 1 (colostrum-deprived, 24 h, group B) (Fig. 5).

Small numbers of coccidia were identified in the ascending colon of animal 3 (conventional plus *C. parvum*, 24 h, group D). Abnormalities were not detected in the rumen of any animal.

Immunohistochemistry and confocal microscopy.

Multifocal, variable-sized colonies of *E. coli* O157:H7 organisms were identified in three of the four animals examined (1, 2 and 4) (Figs 3 and 5). *E. coli* O157:H7 colonies were identified most frequently in the rectum, although *E. coli* O157:H7 colonies were also identified in the caecum and RAJ. Lesions tended to involve one, two or up to several adjacent epithelial cells. Lesions identified in the mucosa at the terminal rectum were found on

Fig. 5. Lamb 1 (colostrum-deprived plus *C. parvum*, group B) 1 day p.i. with *E. coli* O157:H7, ileum. Cryptosporidia (arrows) are present on the epithelial surface. HE staining; bar, 25 µm.

both lymphoid-associated and non-lymphoid-associated epithelium.

Transmission electron microscopy. AE lesions associated with bacteria were confirmed on the intestinal mucosa (Fig. 6). Cryptosporidia were observed in the ileum of one animal (1, colostrum-deprived plus *C. parvum*, group B) (Fig. 7), confirming the light-microscopic findings.

DISCUSSION

It is established that *E. coli* O157:H7 colonizes the intestinal tract of ruminants, often leading to persistent infection (Cray & Moon, 1995; Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1999; Woodward *et al.*, 2003; La Ragione *et al.*, 2005a; Cookson *et al.*, 2002). In a conventional 6-week-old lamb model, it has been previously reported that the intimin of *E. coli* O157:H7 is a significant factor for colonization and persistence, but that AE lesions, for which intimin is essential, are small and extremely sparse in infected animals (Woodward *et al.*, 2003). Interestingly, in the present study, the frequency, density and distribution of *E. coli* O157:H7-induced AE lesions observed in the animals was higher than previously reported.

We have shown previously (Wales *et al.*, 2001b; Woodward *et al.*, 2003) that in conventional lambs of this age, faecal shedding of *E. coli* O157:H7 after oral inoculation declines gradually from 10^7 to 10^3 c.f.u. (g faeces)⁻¹ over the first 10–14 days, and thereafter faecal shedding is intermittent. Similar findings were found in the present study for early sampling times (days 1, 3, 8 and 11 or 12). The experimental differences between this study and others that may have contributed to the observed outcomes were the deprivation of colostrum and ewes' milk, and the pre-inoculation with *C. parvum*.

It has been demonstrated in neonatal calves that colostrum is highly protective against *E. coli* O157:H7 challenge and

Fig. 7. Lamb 1 (colostrum-deprived plus *C. parvum*, group B) 1 day p.i. with *E. coli* O157:H7, ileum. Cryptosporidia (arrows) are attached to the surface of epithelial cells. Transmission electron micrograph; bar, 1 μ m.

that AE lesions are readily induced in the distal GIT if colostrum is withheld (Dean-Nystrom *et al.*, 1997). In conventional, 6-day-old lambs, it has been shown that small and sparse AE lesions are induced by *E. coli* O157:H7 (Wales *et al.*, 2001b). Given these data, it seems reasonable to assume that neonatal lambs deprived of colostrum would be more susceptible to colonization by *E. coli* O157:H7 and extensive AE lesion formation. Of concern, and relevant to this study, was the issue of longer-term sequelae for lambs deprived of colostrum and ewes' milk, such as the hand-reared orphan lambs that are frequently found on petting farms. The lamb model used in this study therefore attempted to mimic this scenario. The experimental evidence indicated that *E. coli* O157:H7-induced AE lesions were detected readily in the distal GIT of lambs deprived of colostrum and ewes' milk, and that faecal shedding of *E. coli* O157:H7 organisms was greater than for conventional animals of the same age. By comparison, in a previous study, in which 6-week-old conventional lambs were challenged with the same *E. coli* O157:H7 strain, and in which a similar number of tissues were examined (Woodward *et al.*, 2003), only two lesions were identified, in a single animal, one in the caecum and one in the rectum. The present data support the concept that colostrum is essential for the longer-term health of the GIT, even beyond weaning (Butler, 1979; Logan *et al.*, 1974).

Following the incidental observation of high faecal shedding of *E. coli* O157:H7 and readily identified multifocal AE lesions associated with *C. parvum* in the large intestine of an experimentally orally inoculated 8-week-old goat kid (La Ragione *et al.*, 2005a), this raised the question of whether *C. parvum* has an effect on *E. coli* O157:H7 colonization and shedding in the lamb model used in this study. Concurrent infections with two or more enteropathogens, including cryptosporidia in association with *E. coli*, in naturally infected diarrhoeic and asymptomatic calves and goats, have been described previously (Moon *et al.*, 1978; Janke *et al.*, 1990; de la Fuente *et al.*, 1999; Gunning *et al.*,

Fig. 6. Lamb 4 (conventional plus *C. parvum*, group D) 4 days p.i. with *E. coli* O157:H7, rectum. AE *E. coli* (arrows) are attached to the surface of epithelial cells. Transmission electron micrograph; bar, 0.5 μ m.

2001). In natural and experimental infections in goats and lambs, *C. parvum* causes severe clinical disease, often with high morbidity and mortality (Tzipori *et al.*, 1981; Angus *et al.*, 1982; Ortega-Mora *et al.*, 1993; Koudela & Bokova, 1997; Johnson *et al.*, 1999; Castro-Hermida *et al.*, 2002), and severe lesions are often induced in the posterior jejunum and ileum (Koudela & Jiri, 1997). However, asymptomatic carriage of cryptosporidia has been described in adult goats (Noordeen *et al.*, 2002; La Ragione *et al.*, 2005a). In the present study, lambs challenged with *C. parvum* remained asymptomatic, but two animals shed cryptosporidia for up to 39 days post-inoculation. On microscopical examination of the four lambs examined post-mortem, cryptosporidia were only found associated with the mucosa of the ileum in one lamb, and not in the large intestine where *E. coli* O157:H7-induced AE lesions were detected. It is well documented that the formation of *E. coli* O157:H7 AE lesions is the result of actin polymerization (Shaner *et al.*, 2005; Knutton *et al.*, 1989), and similarly *C. parvum* utilizes actin polymerization to initiate infection (Elliott & Clark, 2000; Elliott *et al.*, 2001). This may explain the opportunistic co-localization that has been described previously between *E. coli* O157:H7 and *C. parvum* in a goat (La Ragione *et al.*, 2005a). The absence of cryptosporidia in the large intestine may indicate that the model used in this study was not appropriate, especially with regard to the timing of bolus infection with *E. coli* O157:H7, to reproduce the incidental finding observed in the goat study (La Ragione *et al.*, 2005a). However, the relative frequency with which O157:H7-induced AE lesions were observed in one conventional animal inoculated with *C. parvum* prior to *E. coli* O157:H7 in this study may indicate a predisposition within that animal to colonization by *E. coli* O157:H7.

The shedding profiles of *E. coli* O157:H7 NCTC 12900, irrespective of the study group, were broadly similar to each other, and consistent with shedding profiles shown in previous studies (Wales *et al.*, 2001b). The numbers of *E. coli* O157:H7 organisms declined over the first 10–14 days after inoculation, followed by intermittent shedding thereafter for up to 18 days. With the exception of two time points soon after oral inoculation (day 3), at which the lambs inoculated with *E. coli* O157:H7 alone shed more *E. coli* O157:H7 than those pre-dosed with *C. parvum*, there were no statistically significant differences between groups. However, a trend was shown which suggested that colostrum deprivation and prior *C. parvum* challenge increased the number of *E. coli* O157:H7-positive faecal samples compared to the conventional animals. The duration of *E. coli* O157:H7 shedding, however, was not increased. It may be hypothesized that the cellular immune responses evoked by *C. parvum* act in the early stages of *E. coli* O157:H7 infection, hence the observation of lower initial shedding of *E. coli* O157:H7 in the *C. parvum* pre-dosed groups.

Recent studies in cattle reported by Naylor *et al.* (2003) suggest a preferred site of colonization and AE lesion formation by *E. coli* O157:H7 to be the terminal rectum.

Bach *et al.* (2005) have linked this observation with a 'super shedder' status, such that these may act as sentinel animals within a herd. Sheng *et al.* (2004) have confirmed the tropism for *E. coli* O157:H7 colonization of the terminal rectum of cattle by rectal inoculation studies. These authors however, do not cite any data on the colonization of sheep following rectal administration of the inoculum. In the present study, the *E. coli* O157:H7-induced AE lesions were not associated specifically with lymphoid tissue in the terminal rectum, as has been described for cattle (Naylor *et al.*, 2003). Therefore, data from this and previous studies (Wales *et al.*, 2001b, 2005) suggest that there are significant differences between cattle and sheep with regard to tissue tropism during colonization.

Collectively, the data produced in this study suggest that lambs are predisposed to *E. coli* O157:H7 colonization by colostrum deprivation, but the effects of concurrent infection with *C. parvum* are less clear. The number of animals in the study was a limiting factor, and trends, rather than statistically confirmed differences between treatment groups, were observed. These trends need to be examined further before firm conclusions can be drawn. However, the potential public health implications of the findings of this study are of particular importance. Lambs on petting farms are frequently orphans which may not have received adequate colostrum (La Ragione *et al.*, 2005b). A consequence may be greater susceptibility to infection with *C. parvum*, *E. coli* O157:H7 or other gastrointestinal pathogens. It is widely accepted that ruminants are the primary source of *E. coli* O157:H7 and *C. parvum* infections for humans, and so a greater understanding of the relationship between these two enteropathogens may lead to the development of effective intervention strategies.

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