BACTERIAL PATHOGENICITY

Variation in the persistence of *Escherichia coli* O157:H7 in experimentally inoculated 6-week-old conventional lambs

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Six-week-old lambs were inoculated orally with $10^9$ cfu of an antibiotic-resistance marked four-strain mixture of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to investigate faecal excretion and intestinal colonisation. In the first experiment, three *E. coli* O157:H7 isolates were not detected in the faeces of any lambs beyond day 8 post inoculation (pi), or from any of the tissues derived from inoculated animals. One strain, 140065 Nalr, was isolated from the caecum and colon of one lamb on day 9 pi, from the rectum of another on day 22 pi and persisted in the faeces for up to 28 days pi. All animals remained clinically normal throughout the study period and histological evidence of adhesion of *E. coli* O157:H7 to the intestinal mucosa was not found. In a separate experiment, four 6-week-old lambs were inoculated orally with $10^9$ cfu of *E. coli* O157:H7 strain 140065 Nalr alone. Faecal samples were positive for this strain until the end of the experiment (day 19 pi). This strain was also recovered from the gastrointestinal tract of lambs on days 6, 18 and 19 pi, but was not isolated at day 17 pi. When sampled separately, rectum and terminal colon contents contained higher numbers of the inoculated strain than the intestinal tissue at these sites. Animals inoculated with O157:H7 strain 140065 Nalr alone produced soft faeces from day 5 pi onwards. Although attaching and effacing lesions were observed in the caecum, proximal colon and rectum in one animal on day 18 pi, the adherent bacteria did not stain with antisera raised against the O157 antigen.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 has become a major concern in the food industry since its association with human disease in the early 1980s [1, 2]. Infection may lead to diarrhoea, but complications such as haemorrhagic colitis and haemolytic uraemic syndrome may also occur [1]. Carriage of the organism may be asymptomatic, and person-to-person transmission is common [3]. Food-borne infection with EHEC O157:H7 is often associated with the consumption of beef (hamburgers and cold cooked meats) or dairy (unpasteurised milk and cheese) products [4] although EHEC have also been isolated from other foods such as unpasteurised apple juice [5] and mayonnaise [6].

The epidemiological link between human disease and consumption of bovine products has indicated that cattle are a primary reservoir for EHEC O157:H7 [4] although recent studies have indicated that sheep are also an animal reservoir [7–13]. Although the mechanisms that mediate persistence within cattle have not been identified, interaction with the host and other bacteria via surface structures may play a role. For example, adhesion of EHEC to epithelial cells is often associated with regions of microvillus effacement where bacteria are intimately attached to the host cell.
Materials and methods

Bacterial strains and inocula

Human EHEC O157:H7 strains EC157 Strr (streptomycin resistant) and 140065 Nalr (nalidixic acid resistant), and bovine EHEC O157:H7 strains 218 Rifr (rifampicin resistant) and 222 Nalr Rifr have been described previously [34, 35]. Strains EC157 and 218 possessed both stx1 and stx2 toxins and strains 140065 and 222 possessed stx2 only. All four strains generated the localised adherence phenotype on HEp-2 tissue culture cells and generated actin re-arrangements characteristic of the AE lesion, as demonstrated by fluorescence actin staining (FAS) [36]. Inocula were prepared by growing bacteria overnight at 37°C in Luria-Bertani (LB) broth. For the four-strain mixed inoculum, samples (2.5 ml) of each of the overnight cultures of EC157 Strr, 140065 Nalr, 218 Rifr and 222 Nalr Rifr were mixed together and made up to 100 ml with phosphate-buffered saline (PBS, pH 7.4). For the single-strain inoculum, a sample (10 ml) of strain 140065 Nalr was made up to 100 ml with PBS. Therefore, these bacterial suspensions contained c. 108 cfu/10-ml volume that was used as the inoculum. Samples were serially diluted and spread on LB agar plates with appropriate antibiotic supplements to determine viable counts. Selection for EC157 Strr, 140065 Nalr, 218 Rifr and 222 Nalr Rifr from the derived samples was made on CHROMagar O157 (CHROMagar) supplemented with streptomycin (25 µg/ml), nalidixic acid (15 µg/ml), rifampicin (150 µg/ml), or nalidixic acid (15 µg/ml) and rifampicin (150 µg/ml) together.

Animals

Ten 6-week-old weaned cross-bred lambs were maintained in a paddock. Samples of rectal contents were taken on two separate occasions and were subjected to immunomagnetic separation (IMS) after pre-enrichment in buffered peptone water (BPW) [25, 37]. Washed beads were incubated overnight on CHROMagar O157 plates to screen for E. coli O157:H7. All animals were confirmed to be free of E. coli O157:H7 by this method before inoculation.

Inoculation and tissue collection

For the four-strain inoculum, four animals were selected at random, individually tagged with a unique identifier (A–D), sheared on the right flank, and penned together in a biological hazard containment facility. They were given 1 week to adjust to the new environment and were fed a complete pelleted diet with water ad libitum. After 1 week, each animal was inoculated orally with the four-strain mixture of E. coli O157:H7. Animals were observed twice a day and faecal samples from each animal were collected daily for bacteriological analysis. One animal was anaesthetised with intravenous pentobarbitone on each of days
immediately after excision from the animal and left
Tissues were placed in neutral buffered formalin 10%
8
homogenate was diluted 10-fold serially in BPW and
respectively and homogenised. A most probable num-
collected, placed in 9-ml and 22.5-ml volumes of BPW
as follows. Faecal (1 g) and tissue (2.5 g) samples were
Samples were analysed immediately after being taken,
Bacteriological examination

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Results

Clinical findings

Lambs A–D, inoculated with the mixed strains, remained clinically normal throughout the experiment. Their faeces were firm to solid with no evidence of diarrhoea or blood. Lamb E, inoculated with strain 140065 Nalr alone, had mild diarrhoea at 5 days pi and soft faeces on day 6 pi when it was killed. Lambs F–H, also inoculated with E. coli O157:H7 140065 Nalr alone, had soft faeces from day 5 pi until they were killed. There was no loss of appetite or evidence of dehydration in any of the inoculated lambs. The two control lambs were clinically normal at the time they were killed.

Bacteriological findings

Recovery of the four differentially marked E. coli O157:H7 strains from faecal samples collected from lambs A–D is shown in Fig. 1. Although three of the four strains (EC157 Strr, 218 Rifr and 222 NalrRifr) were detectable in faecal samples from all lambs for at least the first 4 days pi, they were not isolated from any faecal sample beyond day 9 pi (Fig. 1). Strain 140065 Nalr persisted at c. 1 × 104 cfu/g of faeces in lamb C (until 21 days pi) and in lamb D (until 28 days pi) but it was not detected in samples from lamb B by day 13 pi (Fig. 1). Of the tissues taken for bacteriological examination from lambs A–D, only 2 of 36 were positive for O157 bacteria. Strain 140065 Nalr was detected in the ascending colon (2 × 105 cfu/g of tissue) and caecum (2.25 × 105 cfu/g of tissue) of lamb A at day 8 pi. Whereas three of the four strains were undetectable by day 9 pi, strain 140065 Nalr persisted for up to 28 days pi. From these data, the criterion for a persistent strain was one that was excreted for >14 days pi.

A second study involved the inoculation of four lambs (E–H) with strain 140065 Nalr alone. Increased levels

Histological examination

Tissues were placed in neutral buffered formalin 10% immediately after excision from the animal and left immersed for at least 24 h at room temperature. Trimmed tissues were processed routinely and embedded in paraffin wax, and 4-μm sections were stained with haematoxylin and eosin (H) for light microscopy. Furthermore, sections of intestinal tissues from lamb G plus a representative sample of intestinal tissues with loosely adherent luminal bacteria from five other animals and the tonsil tissues from lamb H were immunostained by an indirect peroxidase-antiperoxidase (PAP) stain as described previously [35]. The primary antiserum was polyclonal and raised against the E. coli O157 lipopolysaccharide antigen. E. coli O157 and O26 cultures were formalin-fixed, suspended in agar and then embedded in paraffin wax and sectioned at 4 μm These control sections were used to verify the effectiveness and specificity of the immunostaining technique.

For the single-strain inoculum, four animals (E–H) were selected and housed as above. After 1 week, each animal was inoculated orally with E. coli O157:H7 strain 140065 Nalr. Monitoring and faecal sampling were performed as described above. Tissues were sampled under terminal anaesthesia as above from one animal on each of days 6, 17, 18 and 19 pi. In addition to the sample sites used for animals A–D, samples of the intrapelvic portion of the rectum and the contents of the distal large intestine were also collected. Two lambs (1 and 1) were uninoculated controls and tissue samples were collected as above under terminal anaesthesia at 10 weeks of age for histological examination.

All procedures complied with the Animals (Scientific Procedures) Act 1986 and were performed under Home Office Licence 70/4987.

Bacteriological examination

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Histological examination

Tissues were placed in neutral buffered formalin 10% immediately after excision from the animal and left

9, 15, 22 and 29 post inoculation (pi) respectively. Following aseptic preparation of the right flank, a laparotomy was performed and tissue samples comprising rumen, duodenum, jejunum, ileum, caecum, colon (three sites), tonsils, mesenteric lymph nodes, liver, spleen and kidney were collected, by a clean technique, for bacteriological and histological examination. The animal was then killed humanely.

For the single-strain inoculum, four animals (E–H) were selected and housed as above. After 1 week, each animal was inoculated orally with E. coli O157:H7 strain 140065 Nalr. Monitoring and faecal sampling were performed as described above. Tissues were sampled under terminal anaesthesia as above from one animal on each of days 6, 17, 18 and 19 pi. In addition to the sample sites used for animals A–D, samples of the intrapelvic portion of the rectum and the contents of the distal large intestine were also collected. Two lambs (1 and 1) were uninoculated controls and tissue samples were collected as above under terminal anaesthesia at 10 weeks of age for histological examination.

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Bacteriological examination

Samples were analysed immediately after being taken, as follows. Faecal (1 g) and tissue (2.5 g) samples were collected, placed in 9-ml and 22.5-ml volumes of BPW respectively and homogenised. A most probable number approach was used to determine, semi-quantitatively, the bacterial count in each sample. Thus, each homogenate was diluted 10-fold serially in BPW and then each dilution was incubated for 6 h at 37°C before 1 ml was processed by immunomagnetic separation (IMS). Briefly, 20 μl of beads (Dynal) coated in polyclonal antisera raised against the O157 lipopolysaccharide antigen. E. coli O157 and O26 cultures were formalin-fixed, suspended in agar and then embedded in paraffin wax and sectioned at 4 μm These control sections were used to verify the effectiveness and specificity of the immunostaining technique.
Days after inoculation

Approximate log10 cfu/g faeces

D

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

A

B

C

D

Days after inoculation

Fig. 1. Faecal excretion of *E. coli* O157:H7 strains from lambs A–D as measured by immunomagnetic separation (IMS) and plating on to CHROMagar O157 supplemented with antibiotics. ■, EC157 Strr; ○, 140065 Nafr; ▲, 218 Rifr; ●, 222 Nafr Rifr.

VARIATION IN PERSISTENCE OF E. COLI O157:H7 IN LAMBS
of shedding were associated with lambs inoculated with strain 140065 Nalr alone, compared with the same strain in the mixed inoculum (Figs. 1 and 2). Strain 140065 Nalr was recovered from daily faecal samples from all lambs until day 6 pi, when lamb E was killed to determine whether the mild diarrhoea and soft faeces were associated with any pathological changes. Bacteria were recovered (10^2–10^4 cfu/g of tissue) from lamb E throughout the alimentary tract from rumen to rectum (Table 1). Intermittent excretion of strain 140065 Nalr was observed in lamb F. It was not isolated from faeces on days 10, 11, 13 or 17 pi, or from any tissue sites when the animal was killed at day 17 pi (Fig. 2b, Table 1). Bacteria were recovered daily from the faeces of lamb G until it was killed on day 18 pi. On examination of the tissues from this lamb, strain 140065 Nalr was recovered only from the contents of the terminal colon (<10^3 cfu/g tissue). Strain 140065 Nalr was recovered in high numbers (>10^6 cfu/g) from the faeces of lamb H (Fig. 2d) and was recovered from the ileum through to the rectum in high numbers (10^7–10^9 cfu/g) (Table 1). Higher numbers of bacteria were isolated from both the rectal contents (lamb E and H) and terminal colon contents (lamb E, G and H), than were isolated from the intestinal wall samples at the respective sites (Table 1).

Macroscopic pathological findings

Significant changes were not observed in any animal at any site examined.

Histopathological findings

With H&E staining, 16 sections of rumen, 82 sections of small intestine and 219 sections of large intestine were examined from the inoculated animals. Preservation of the intestinal mucosa was generally excellent or good. Mild autolytic changes were present at about half of the sites examined and comprised detachment and loss of epithelium from a minority of villus tips in the small intestine, and small areas of epithelial detachment and single epithelial cell loss at the luminal surface in the large intestine. Four sites in the large intestine demonstrated more marked autolysis in some areas.

In the caecum, proximal loop of the ascending colon and rectum of one animal (lamb G), multiple foci of attaching-effacing bacteria were found. In several large and small intestine sections, loosely adherent luminal contents including bacteria were present on the mucosal surface. AE lesions were not observed in the control animals.

A number of features were noted in tissues from both the control and the inoculated animals. These included small numbers of coccidia in the small and large intestines and slender organisms of spirochaetal morphology in some large intestine crypts. Encysted and luminal nematodes and small crypt abscesses were occasionally present in some sections from inoculated animals. The tonsillar crypts of control and inoculated animals contained adventitious vegetable matter with accompanying bacteria and inflammatory cells. Significant changes were not seen in the liver, kidney or spleen of any animal.

Immunohistochemistry

The O157 antiserum proved sensitive and specific for E. coli O157 when used to stain control sections positive for E. coli O157 and O26. The immunostained sections from lamb G included AE lesions. Positive staining of bacteria was not observed in any section.

Discussion

Several studies have established that sheep are a reservoir for E. coli O157:H7 [7, 8, 11–13, 33]. The present study demonstrated that 6-week-old weaned lambs are a suitable model for investigating the persistence of a mixed strain inoculum of E. coli O157:H7, and that one strain was continually excreted for up to 28 days. The numbers of O157:H7 excreted were determined semi-quantitatively and, although recognised by the authors to be an estimate only, strain 140065 Nalr was excreted in high numbers (>10^4 cfu/g of faeces) consistently in one animal. The limited number of animals and modest scope of bacteriological analysis in each experiment was imposed by the category III containment required. Therefore, the data are not amenable to rigorous statistical analysis. However, the two experiments gave similar persistence data for strain 140065 Nalr and it is of interest that this persistently excreted strain was derived from a human source with established human pathogenicity (Henry Smith, personal communication). When given a mixed strain inoculum, none of the animals exhibited any clinical symptoms for the duration of the experiment. However, mild diarrhoea or the intermittent production of soft faeces was noted in lambs inoculated with the single strain 140065 Nalr, proven in its persistence. The clinical signs in these lambs may be associated with the four-fold increase in the dose of the persistent 140065 Nalr isolate, when compared with the asymptomatic lambs inoculated with the four-strain mixture. No other clinical abnormalities were detected throughout the study.

Previous studies have noted the formation of AE lesions after experimental inoculation of O157:H7 into gnotobiotic piglets [31] and neonatal calves (<36 h old) [30]. Naturally occurring [38] and experimental [39] non-O157 AE lesions have been reported in neonatal lambs, and E. coli O157:H7 induced very small and sparse AE lesions in 6-day-old colostrum-fed lambs [35]. In 6-month-old sheep, association of E. coli O157 with the mucosa was not observed even in an
Fig. 2. Faecal excretion of *E. coli* O157:H7 140065 Nal⁺ strains from lambs E–H as measured by immunomagnetic separation (IMS) and plating on to CHROMagar O157 supplemented with nalidixic acid.
animal that showed persistent colonisation by the organism [34]. Thus, one aim of the present study was to establish whether AE lesion formation was associated with persistence in lambs of 6–10 weeks of age. Although persistent infection was established in this age group, AE lesions attributable to E. coli O157:H7 were not observed. This is consistent with the findings of other investigators [22, 30, 32] who showed persistent colonisation of streptococcal and nalidixic acid-resistant double mutants in their studies although the doses to effect this were at least one order of magnitude higher than those used in the studies reported here. Whether the dose compensates for possible attenuation requires examination.

Several surveys have noted a lower prevalence of O157 in sheep than cattle [4, 7, 10, 40], although the role of the AE lesion in persistent colonisation of cattle is unknown. It is also possible that isolates of O157:H7 derived from sheep may colonise the ovine gastrointestinal tract more efficiently than the bovine and human isolates that were used in this experiment. Data from the present study are also consistent with the possibility that long-term persistence of E. coli O157:H7 in sheep may be mediated in part by bacterial multiplication within the luminal contents of the gastrointestinal tract rather than adherence. Indeed, at several sites (terminal colon and rectum) of lambs E, G and H, greater numbers (100-fold) of strain 140065 Nalr were isolated from luminal contents than from the corresponding intestinal wall samples.

The present study demonstrated considerable differences in the colonisation and persistence of inoculated EHEC O157:H7, both between bacterial strains and between individual animals. No correlation was observed between the strain characteristics determined in vitro and the persistence behaviour observed in vivo. Possible causes of this include competition between strains – although there was no evidence of this for pairs of strains co-inoculated in vitro (data not shown), a differential effect of antibiotic resistance labelling, and inherent strain variability with respect to persistence factors that are currently unknown. One of three animals given the mixed strain inoculum was no longer excreting strain 140065 Nalr by 14 days pi. Another lamb, which was inoculated with strain 140065 Nalr only, did not have a detectable level of the inoculum strain in the faeces on days 10, 11, 13 and 17 pi, despite it being present on the intervening days. This intermittent excretion of O157:H7 suggests that it may be almost impossible to determine whether cattle or sheep are free of O157:H7 by current detection methods unless a daily faecal sampling series is performed. However, one lamb consistently shed strain 140065 Nalr in higher numbers than all the others, which indicates a role for host factors, currently

Table 1. Tissue isolation of E. coli O157:H7 140065 Nalr from lambs E–H

<table>
<thead>
<tr>
<th>Tissue</th>
<th>E</th>
<th>F</th>
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<tr>
<td>Tonsil</td>
<td>ND</td>
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<tr>
<td>Rumen</td>
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<td>ND</td>
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<tr>
<td>Illeum</td>
<td>2</td>
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<tr>
<td>Caecum</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Terminal colon</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>Tissue contents</td>
<td>4</td>
<td>ND</td>
<td>&lt;3</td>
<td>6</td>
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<tr>
<td>Rectum</td>
<td>2</td>
<td>ND</td>
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<td>4</td>
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<tr>
<td>Tissue contents</td>
<td>4</td>
<td>ND</td>
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ND, not detected.

*K*By immunomagnetic separation and plating on to CHROMagar O157 supplemented with nalidixic acid.
 Unknown, in the level and possibly the duration of shedding of E. coli O157:H7 by sheep.

In the present study, isolation of E. coli O157:H7 from the gastrointestinal tract of animals inoculated with the mixed strain inoculum was rare and limited to the distal regions only. However, when inoculated alone, strain 140065 Nal1 was isolated throughout the gastrointestinal tract of both lambs. Thus, it is possible that there may have been some reduction of colonisation in animals given the mixed strain inoculum rather than the persistent strain 140065 Nal1 alone, but the possible mechanisms for this are unknown.

The present study indicated that the technique of immunomagnetic separation can be used to isolate O157:H7 strains from tissue and fecal samples. This method has been used successfully to isolate EHEC O157:H7 from raw meat products containing <10 cfu/g [37]. However, on several occasions strain 140065 Nal1 was undetectable in the intestinal tissue samples when it was still being isolated from faeces. We are unaware of other studies that have used IMS to isolate small numbers of EHEC O157:H7 directly from animal tissue, but loss of beads because of the direct hydrophobic interaction of tissue and beads may reflect the reduced sensitivity of the IMS technique for isolation of E. coli O157 from tissues and intestinal contents, compared with faeces.

In summary, this study demonstrated the persistence of E. coli O157:H7 for up to 28 days in an ovine model with differentially marked isolates. Faecal culture demonstrated that certain O157:H7 strains may be excreted for longer periods than others, and that host factors such as age and diet appear to affect the duration of excretion. Mucosal association by the inoculated strains, including AE lesions, was not identified in any of the inoculated animals.

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