

# Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine

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## Abstract

**Background**—Enterohaemorrhagic *Escherichia coli* (EHEC) constitute a significant risk to human health worldwide, and infections, particularly with serogroup O157:H7, are associated with consumption of a variety of food and water vehicles, particularly food of bovine origin. EHEC cause acute gastroenteritis, bloody diarrhoea, and haemorrhagic colitis; up to 10% of cases develop severe complications, including the haemolytic uraemic syndrome, with a 5% case fatality. A virulence characteristic of enteropathogenic *E coli*, the attaching/effacing lesion, is considered to be important in EHEC. However, although EHEC produce this lesion on cultured human cells, this has not been demonstrated on human intestinal mucosal surfaces. In addition, the initial site(s) of colonisation of EHEC in humans is not known.

**Aims**—To assess the association of EHEC O157:H7 with paediatric and bovine intestine using *in vitro* organ culture and determine if attaching/effacing lesions occur.

**Methods**—Ultrastructural analysis of *in vitro* intestinal organ cultures of human small and large intestine was used to investigate adhesion of O157:H7 EHEC to intestinal surfaces. Bovine intestinal organ culture was used to examine the pathology produced by the same EHEC strain in cattle.

**Results**—The study showed that EHEC O157:H7 adhered to human intestinal mucosa. Binding and attaching/effacing lesion formation of O157:H7 in humans was restricted to follicle associated epithelium of Peyer's patches. The same strain caused attaching/effacing lesions on bovine mucosa.

**Conclusions**—O157:H7 targets follicle associated epithelium in humans where it causes attaching/effacing lesions. The same human isolate can cause attaching/effacing lesions in cattle, indicating that similar pathogenic mechanisms operate across human and bovine species

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Enterohaemorrhagic *Escherichia coli* (EHEC) comprise a group of emerging zoonotic pathogens of worldwide importance.<sup>1</sup> The most prominent serotype within the EHEC group is O157:H7.<sup>2</sup> In humans, EHEC cause acute gastroenteritis, bloody diarrhoea, and haemorrhagic colitis and are responsible for serious disease outbreaks associated with contaminated food. Up to 10% of cases develop severe complications including haemolytic uraemic syndrome (HUS) with a case fatality rate of approximately 5%.<sup>1-3</sup> The systemic diseases associated with EHEC infections are almost certainly linked to expression and translocation of the bacteriophage associated shiga toxin (Stx) across the gut epithelium.<sup>4,5</sup> However, additional virulence factors, such as the attaching/effacing (A/E) phenotype<sup>6,7</sup> and possession of the pO157 plasmid are required for expression of full virulence.<sup>8</sup> Indeed, it has been shown that an O157:H7 strain depleted of the bacteriophage encoded Stx is as pathogenic, in terms of diarrhoeal disease, as the parent EHEC strain in animal models.<sup>9</sup>

EHEC, in common with enteropathogenic *E coli* (EPEC), possess the ability to form characteristic A/E lesions on mammalian cells growing in culture<sup>10</sup> and in animals.<sup>11,12</sup> The first gene to be associated with A/E activity was the EPEC *eae* gene encoding intimin, an outer membrane protein adhesin essential for intimate bacterial attachment to eukaryotic host cells.<sup>13</sup> The *eae* gene has also been detected in EHEC O157<sup>14</sup> and other EHEC serogroups,<sup>15</sup> and has been shown to be required for colonisation in newborn piglets<sup>5</sup> and calves.<sup>7</sup> However, there are distinct differences between EPEC and EHEC making it important to compare their interaction with the intestine. At least four distinct intimin types, designated  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , have been identified,<sup>16</sup> and importantly, different intimin types were found to be preferentially associated with defined EPEC and EHEC serotypes. These may confer different tissue targeting properties during infection.<sup>17</sup> All of the genes necessary for A/E

**Abbreviations used in this paper:** EHEC, enterohaemorrhagic *Escherichia coli*; A/E, attaching/effacing; HUS, haemolytic uraemic syndrome; EPEC, enteropathogenic *E coli*; LEE, locus of enterocyte effacement; Esps, EPEC secreted proteins; Tir, translocated intimin receptor; PER, plasmid encoded regulator; IVOC, *in vitro* organ culture; SEM, scanning electron microscopy; TEM, transmission electron microscopy; FAE, follicle associated epithelium; Stx, shiga toxin.

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lesion formation map to a pathogenicity island termed the locus of enterocyte effacement (LEE), and include structural components of a type III secretory apparatus, secreted proteins (EspA, EspB, EspD), the translocated intimin receptor (Tir), and their respective chaperones (reviewed by Frankel and colleagues<sup>18</sup>). Recently it was shown that the LEE region from O157:H7 was unable to confer the attaching/effacing phenotype or secrete Esps when cloned in a laboratory *E coli* strain background, indicating that there are functional and regulatory differences between EPEC and EHEC LEE regions.<sup>19</sup> Other differences are that O157:H7 does not produce a tyrosine phosphorylated Tir<sup>20</sup> and it takes longer to produce A/E lesions on HEp-2 cells.<sup>10</sup>

Cattle represent an important reservoir of EHEC, and human infection with contaminated bovine food products represents a major cause of food borne infection. The bacterial and host factors influencing colonisation and pathogenicity in cattle and humans remain poorly defined. Conventionally reared cattle are generally considered to be resistant to natural infection with O157:H7 but a carriage rate of up to 4% in healthy cattle has been reported.<sup>21</sup> However, acute outbreaks of severe diarrhoea in cattle infected with this serotype have been described.<sup>22, 23</sup> Moreover, *E coli* O157:H7 can be virulent in neonatal calves<sup>7</sup> but calves older than three weeks of age show only mild symptoms of pyrexia and diarrhoea following oral challenge.<sup>24</sup>

Although EHEC O157:H7 is believed to colonise the human large intestinal mucosa,<sup>1</sup> association of EHEC O157:H7 and A/E lesion formation on human tissue has thus far not been demonstrated. In this *in vitro* study we investigated the interaction of EHEC O157:H7 with human mucosa and tested whether the same strain could cause A/E lesions on bovine intestinal mucosa.

## Methods

### BACTERIAL STRAINS

EPEC E2348/69 (O127:H6)<sup>25</sup> and a spontaneous Stx negative derivative of EHEC 84-289 (O157:H7, termed 85-170)<sup>26</sup> were used throughout. The EHEC strain was originally isolated from a food handler in a Canadian nursing home, is fluorescent actin staining test<sup>10</sup> positive on cultured HEp-2 cells (data not

shown), and expresses a surface located  $\gamma$ -type intimin. Bacteria were stored routinely at  $-70^{\circ}\text{C}$  using a Microbank system (Prolab Diagnostics, Neston, UK). Prior to adhesion studies the strains were subcultured into brain heart infusion broth and incubated aerobically overnight at  $37^{\circ}\text{C}$  without agitation.

### TISSUE SAMPLES

After fully informed parental consent and local ethics committee approval, human tissue was obtained during routine investigation of patients for potential intestinal disorders. Proximal small intestinal mucosa (fourth part duodenum or duodenojejunal flexure) was taken using either a double port paediatric Crosby capsule or grasp biopsy forceps during upper endoscopy (Olympus PCF paediatric endoscope). These patients were being investigated for abdominal pain, chronic diarrhoea, and/or failure to thrive, but no gastrointestinal cause for their complaint was determined. Terminal ileum, Peyer's patches, and transverse colon were taken using the grasp biopsy method during routine lower endoscopy. Peyer's patches can be recognised and selectively biopsied during endoscopy,<sup>27</sup> a technique made easier by the application of video endoscopy. Patients undergoing lower endoscopy either had no cause found for their presenting symptoms of abdominal pain and/or bloody diarrhoea or had inflammatory bowel disease or a polyp diagnosed, but no involvement of the region biopsied for this study was detected on gross or microscopic observation. Median age and age range of the patients are shown in table 1. All endoscopic biopsies were taken from areas showing no obvious pathology or other abnormality, and all intestinal histology was reported to be normal in the material used in this study.

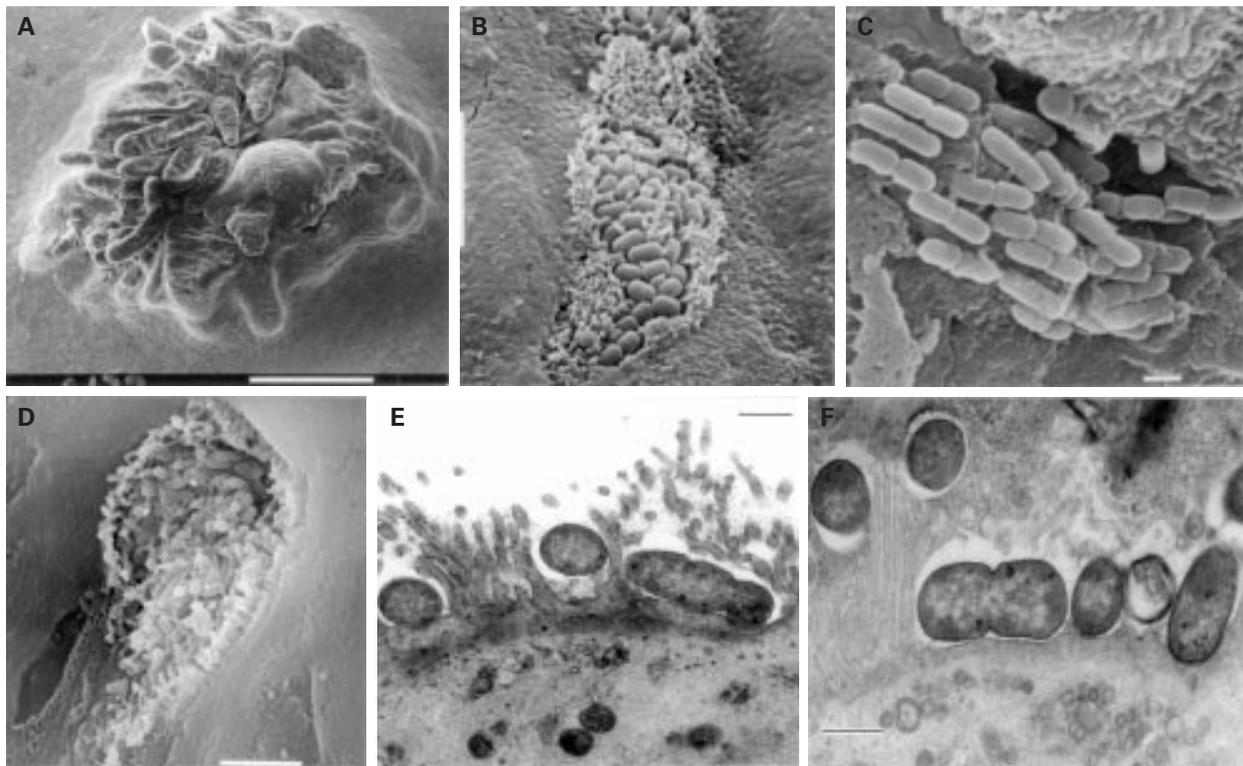
Bovine tissue was obtained from an adult Friesian cow undergoing routine slaughter at the Institute for Animal Health (Compton, Berkshire, UK). The cow had no recent history of diarrhoeal disease. A laparotomy was performed immediately after termination and a full thickness biopsy was taken from the continuous Peyer's patch region in the distal ileum and held at room temperature in *in vitro* organ culture (IVOC) medium<sup>28</sup> for transportation to the laboratory.

### ORGAN CULTURE ADHESION ASSAY

IVOC was performed as described previously<sup>28</sup> for eight hours. Briefly, 25  $\mu\text{l}$  of the overnight bacterial culture was inoculated onto the intestinal sample which had been placed mucosal surface up on a foam support in tissue culture medium (Dulbecco's modified Eagle's medium (with glucose and sodium bicarbonate), NCTC-135 (with L-glutamine), newborn calf serum, and 0.5% D-mannose (Sigma Chemical Co, Dorset, UK)). Each bacterial strain was examined in human IVOC on at least three occasions using tissue from different children. For bovine IVOC, three biopsies were used. An uninoculated specimen was included with each experimental culture to exclude the possibility of *in vivo* bacterial colonisation. After incubation,

Table 1 Patient age and number of enterohaemorrhagic *Escherichia coli* (EPEC), strains O127:H6 (E2348/69) and O157:H7 (85-170), that adhered to *in vitro* organ culture prepared from proximal small intestine, distal small intestine, Peyer's patches, and colonic samples

	Proximal small intestine	Distal small intestine	Peyer's patch	Colon
O127:H6 (E2348/69)				
Patient age (months) (median (range))	101 (41-178)	100 (18-193)	131 (51-180)	136 (53-184)
No of samples	8	10	8	8
No of samples with adhering bacteria (%)	8 (100%)	8 (80%)	6 (75%)	2 (25%)
O157:H7 (85-170)				
Patient age (months) (median (range))	41 (18-109)	123 (30-185)	87 (35-145)	75 (62-191)
No of samples	4	7	5	4
No of samples with adhering bacteria (%)	0	0	5 (100%)	0



**Figure 1** (A)–(E) are of human intestine. (A) *In vitro* organ culture (IVOC) sample showing presence of dome shaped lymphoid follicle (scanning electron microscopy (SEM); bar=500  $\mu$ m). (B) Colony of EPEC E2348/69 on follicle associated epithelium (FAE) (SEM; bar=5  $\mu$ m). (C) EHEC strain 85-170 adhering to FAE in “log-jam” pattern (SEM; bar=1  $\mu$ m). (D) Colony of EHEC strain 85-170 on FAE (SEM; bar=5  $\mu$ m). (E) Attaching/effacing lesion of EHEC strain 85-170 on FAE (transmission electron microscopy (TEM); bar=0.5  $\mu$ m). (F) EHEC strain 85-170 showing attaching/effacing lesion on bovine ileum (TEM; bar=0.5  $\mu$ m).

IVOC specimens were washed thoroughly three times with phosphate buffered saline to remove any non-adherent bacteria and then prepared for electron microscopy as described previously.<sup>28</sup> Samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, and dehydrated in 2,2 dimethoxy-propane. For scanning electron microscopy (SEM), specimens were transferred to absolute ethanol, critically point dried using liquid carbon dioxide in an Emitech K850 apparatus, mounted on aluminium stubs, sputter coated with gold-palladium using a Polaron E5100 sputter coater, and viewed in a JEOL 5300 scanning EM at an accelerating voltage of 30 kV. For transmission electron microscopy (TEM), post-fixed specimens were dehydrated in 2,2-dimethoxypropane with three changes over nine minutes and embedded in TAAB resin (TAAB Laboratories, Reading, UK). Ultrathin sections (0.1  $\mu$ m) were double stained with 2% uranyl acetate and lead citrate and examined in a JEOL 1200-EX II transmission electron microscope at an accelerating voltage of 80 kV.

## Results

### INTERACTION OF EHEC AND EPEC STRAINS WITH IVOC PREPARED FROM HUMAN GUT

The interaction of EHEC strain 85-170 with freshly biopsied human tissues, obtained from different sites within the gut, was examined and compared with the prototype EPEC strain E2348/69. In human gut IVOC assays, the domed mucosal surface overlying individual

lymphoid follicles within Peyer’s patches can be easily recognised in the scanning EM (fig 1A) so that bacterial adhesion to follicle associated epithelium (FAE) can be discriminated from adhesion to other epithelial sites. EPEC strain E2348/69 adhered to IVOC prepared from proximal small intestine, distal small intestine, and FAE of Peyer’s patches (fig 1B) but showed limited adhesion to colonic samples (table 1). A/E lesions were readily detected on IVOC samples infected with EPEC 2348/69 and the location of these lesions correlated with the adhesion pattern. In marked contrast, EHEC 85-170 were not detected adhering to the proximal small intestine, distal ileum, or transverse colon but were detected adhering to FAE in all Peyer’s patch IVOC (fig 1C, D, table 1). Bacteria could not be detected adhering to the epithelium of villi neighbouring the follicle. Some bacterial colonies on FAE displayed a closely arranged parallel array of bacteria (fig 1C), although others were not as organised. Transmission EM identified A/E lesion formation in these regions (fig 1E). FAE associated adhesion appeared to be randomly scattered over the FAE, and definite examples of adhesion to M cells were not recognised. Indeed, M cells were infrequently detected by SEM.

### INTERACTION OF EHEC STRAIN 85-170 WITH IVOC PREPARED FROM BOVINE ILEUM

As cattle are a known reservoir of human *E. coli* O157 infection it was of interest to see if EHEC strain 85-170 could attach to bovine tissues

and form A/E lesions similar to those seen in the human samples. The interaction of EHEC strain 85-170 with cattle tissues was examined using a mucosal sample taken from the continuous Peyer's patch region in the distal ileum and held at room temperature in IVOC medium for transportation to the laboratory. Bacteria were detected binding to the surface of the tissue by microscopy and formed typical A/E lesions (fig 1F). The target cells on the bovine mucosa to which the bacteria adhered could not be clearly identified, although the microvillous length suggested that they were absorptive epithelial cells.

### Discussion

In the present study we have shown for the first time, using IVOC, that *E coli* O157:H7 can attach to and cause A/E lesions on human intestinal mucosal, and that the same strain causes A/E lesions in conventional adult cattle intestine. These novel observations provide a potential link between mechanisms of intestinal colonisation operating across the two species, a factor that may contribute to the interspecies spread of infection. The success in visualising these phenomena in human tissue is probably due to several factors, including the short term nature of the organ culture (eight hours), choice of Peyer's patch material, and absence of Stx encoding genes in the O157:H7 strain used. Under in vivo conditions, patients would only present when symptoms are well advanced and the effects of Stx (including epithelial destruction<sup>29</sup>) are apparent, thus precluding identification of bacterial adhesion and A/E lesion formation; postmortem material is inadequate for ultrastructural analysis of the surface epithelium. Using IVOC of human biopsies, we found that adhesion was limited to the FAE of Peyer's patches and that the pattern of adhesion showed similarities to the "log-jam" pattern described for O157:H7 in cell culture experiments.<sup>30</sup> The apparent discrepancy between O157:H7 adhesion to HEp-2 cells but not to most human intestinal areas, is a previously reported phenomenon for several mutant EPEC strains which readily bind to HEp-2 cells but show no adhesion to paediatric intestine.<sup>28</sup> This may result from the complex brush border in humans versus the morphologically simple microvillous-like processes seen on HEp-2 cells. Our initial studies in cattle served to demonstrate that a human isolate of O157:H7 could cause an A/E lesion in both species. Any comment on the pattern and sites of preferential adhesion in cattle would be premature and must await a more detailed investigation.

Several other bacterial pathogens are believed to exhibit preferential initial adhesion to FAE of Peyer's patches. This phenomenon has been described for human pathogens in animal models, for example *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri*.<sup>31</sup> *Vibrio cholerae* has been shown to adhere to formalin fixed human Peyer's patch epithelium<sup>32</sup> but studies using unfixed human tissues have been limited. We previously demonstrated bacteria adhering to a proximal

intestinal mucosal lymphoid follicle in a child with chronic diarrhoea and a *Salmonella* infection,<sup>33</sup> raising the possibility of FAE involvement in long term bacterial colonisation. Our study shows that Peyer's patches may be involved in the initial events of EHEC colonisation. The specificity of this adhesion is uncertain as Peyer's patches are considered to be sites of antigen sampling. However, several bacteria express adhesins promoting binding to these regions (for example, long polar fimbriae in *Salmonella*,<sup>34</sup> AF/R1 in RDEC-1,<sup>35</sup>  $\beta$ -1 integrin binding in *Yersinia*),<sup>36</sup> and strains not expressing these adhesins show reduced Peyer's patch adhesion and less virulence.<sup>34-37</sup>

Our observations with human IVOC parallel those obtained using a natural EPEC infection (strain RDEC-1) in rabbits. This strain initially colonises FAE of rabbit Peyer's patches and then spreads to other intestinal regions; there is a time lag of 6–7 days between inoculation and development of diarrhoea, with Peyer's patch colonisation occurring within 24 hours, and ileal and colonic colonisation following 2–3 days later.<sup>38</sup> The incubation period for O157:H7 is similar at approximately seven days.<sup>39</sup> We would conclude from our in vitro observations that colonisation of other intestinal regions by O157:H7 is a subsequent event following colonisation of FAE. Certainly, histological studies in humans show extensive damage to colonic mucosa in the form of a haemorrhagic colitis,<sup>40</sup> although the relative contributions of Stx circulating within capillaries and bacteria adhering directly to the mucosal surface (causing A/E lesions and releasing toxin) in causing this damage is uncertain. The infective dose for O157:H7 can be small<sup>41</sup> and this may appear surprising if the initial target of colonisation is restricted to FAE. However, *Shigella* species also have a small infective dose and show preferential adhesion to FAE of Peyer's patches.<sup>42</sup> Both organisms are acid resistant,<sup>43</sup> and this would assist passage through the stomach; growth would occur during transit to the ileocaecal region, where Peyer's patches are common, and reduced motility at the ileocaecal valve would also assist colonisation in this region.

We could not unequivocally demonstrate EHEC O157:H7 binding to M cells as a potential mechanism for selective FAE binding in humans. However, recognition may be difficult as microvillous appearance is altered at sites of A/E lesion formation. M cells, unlike other epithelial cells, may express surface associated  $\beta$ -1 integrins<sup>36</sup> (and possibly other integrins). Intimin, a protein essential for *E coli* O157:H7 colonisation in animal models,<sup>6,7,44</sup> can bind  $\beta$ -1 integrins in vitro<sup>45</sup> and thus could target EHEC to M cells. However, these cells were infrequently seen by SEM in FAE, a method routinely used to identify M cells, and a more formal study is needed to establish their frequency and relevance in humans. A particular intimin derivative, intimin  $\gamma$ , is associated with O157:H7 and related serotypes<sup>16</sup>; replacing the *eae* gene (encoding intimin  $\gamma$ ) of O157:H7 with *eae* (encoding intimin  $\alpha$ ) results in colonisation spreading from the colon to the

small intestine in gnotobiotic piglets.<sup>17</sup> This raises the possibility that intimin  $\gamma$ , whether M cells are involved or not, mediates the preferential binding of O157:H7 to FAE of Peyer's patches, and studies pursuing this line of investigation are underway.

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