Escherichia coli O157:H7 colonisation in small domestic ruminants

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 was first implicated in human disease in the early 1980s, with ruminants cited as the primary reservoirs. Preliminary studies indicated cattle to be the sole source of *E. coli* O157:H7 outbreaks in humans, however further epidemiological studies soon demonstrated that *E. coli* O157:H7 was widespread in other food sources and that a number of transmission routes existed. More recently, small domestic ruminants (sheep and goats) have emerged as important sources of *E. coli* O157:H7 human infection, particularly with the widespread popularity of petting farms and the increased use of sheep and goat food products, including unpasteurised cheeses. Although the colonisation and persistence characteristics of *E. coli* O157:H7 in the bovine host have been studied intensively, this is not the case for small ruminants. Despite many similarities with the bovine host, the pathobiology of *E. coli* O157:H7 in small domestic ruminants does appear to differ significantly from that described in cattle. This review aims to critically review the current knowledge regarding colonisation and persistence of *E. coli* O157:H7 in small domestic ruminants, including comparisons with the bovine host where appropriate.

Contents

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a zoonotic enteric pathogen of worldwide importance. In the early 1980s, human infection with *E. coli* O157:H7 was first recognised to be associated with bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombotic-thrombocytopaenic purpura (Karmali *et al.*, 1983; Riley *et al.*, 1983; Pai *et al.*, 1984; Ryan *et al.*, 1986). 'EHEC' is a pathotype designation that was created for *E. coli* of any serotype that was associated with this pattern of bloody diarrhoea and systemic pathology, including HUS, in humans (Levine, 1987). Subsequently, a typical pattern of virulence determinants in EHEC isolates was established, including the elaboration of Shiga-toxin(s) and the ability to adhere intimately to cell lines *in vitro*, forming attaching-effacing (AE) lesions (Nataro & Kaper, 1998). The first recorded outbreaks of *E. coli* O157:H7 infection in humans were associated with the consumption of ground beef, and thus cattle were soon recognised as important reservoirs (Martin *et al.*, 1986; Bopp *et al.*, 1987; Borczyk *et al.*, 1987; Riley, 1987).

In recent years, the annual incidence of reported clinical EHEC O157:H7 infection in humans in the USA has been around 1 per 100,000 (Centers for Disease Control and Prevention, 2008), with 2621 cases reported in 2005. Twenty-six countries (principally European but including Japan) reported a total of 2,937 EHEC O157:H7 infections to Enternet in 2005 (Anon., 2007). Of these cases, 21% developed HUS, which is a high percentage, suggesting that many less severe cases went unreported.

Transmission of *E. coli* O157:H7 to humans is principally via contamination of food by animal faeces, with cattle considered to be the primary reservoir (Griffin & Tauxe, 1991; Zhao *et al.*, 1995; Hancock *et al.*, 1997). Early studies by Doyle and Schoeni (1987) indicated that *E. coli* O157:H7 was also widespread in meat sources other than beef. In addition, numerous human disease outbreaks have been associated with the consumption of plant products, including apple cider and vegetables such as lettuce, radishes, alfalfa sprouts and spinach (Besser *et al.*, 1993; Fukushima *et al.*, 1999; Ferguson *et al.*, 2005; Centers for Disease Control and Prevention, 2006; Maki, 2006). There are also traceable links between human infection and ruminant faeces via water or direct contact (Licence *et al.*, 2001; Strachan *et al.*, 2001), and evidence that contact with animal faeces is a strong risk factor for sporadic *E. coli* O157:H7 infection (Locking *et al.*, 2001). Interpersonal spread can also be a significant factor in outbreaks (Ryan *et al.*, 1986; Cryan, 1990). Therefore, *E. coli* O157:H7 may also be considered as an environmental pathogen (Strachan *et al.*, 2006; Solecki *et al.*, 2007).

The prevalence of *E. coli* O157:H7 in cattle has been the subject of much research, with most studies concluding that at any given time the majority of cattle are negative for *E. coli* O157:H7 (Zhao *et al.*, 1995; Gansheroff & O'Brien, 2000; Laven *et al.*, 2003; Omisakin *et al.*, 2003). The prevalence quoted in the literature of *E. coli* O157 in individual cattle varies considerably, with rates of 1.8% in Japan (Miyao *et al.*, 1998), 1.9% in Australia (Cobbold & Desmarchelier, 2000), 1.5% in Brazil (Cerqueira *et al.*, 1999), 0 to 7.4% in the USA (Faith *et al.*, 1996) and 1 to 4.2% in England and Wales (Chapman *et al.*, 1993; Richards *et al.*, 1998; Paiba *et al.*, 2003). In Scotland, Synge and Paiba (2000) reported that in beef herds the individual and herd-level prevalences were around 8.7 and 24%, respectively. Differing sampling techniques have hampered valid comparisons between geographical locations and consequently there is some controversy over prevalence rates.

In cattle in England and Wales, Paiba *et al*. (2002) found that the highest prevalence of *E. coli* O157:H7 was seen in late summer and early autumn, which is in agreement with

other sentinel studies (Chapman *et al.*, 1997; Hancock *et al.*, 1997; Tutenel *et al.*, 2002). However, by contrast, Ogden *et al*. (2004) found that the prevalence of *E. coli* O157:H7-positive cattle was higher in the cooler months, although heaviest shedding of the organism by individual cattle was seen in the summer.

Much work to date on the colonisation, persistence and control of *E. coli* O157:H7 has focused on cattle, they being the perceived primary reservoir host. Numerous *E. coli* O157:H7 factors for bovine colonisation have been identified and characterised. However, several other species including rabbits, deer, water buffalo, pigs, chickens and seagulls have also been implicated as carriers of *E. coli* O157:H7 (Griffin & Tauxe, 1991; Pritchard *et al.*, 2001; Eriksson *et al.*, 2003; Galiero *et al.*, 2005; Dipineto *et al.*, 2006; Foster *et al.*, 2006; Scaife *et al.*, 2006; Garcia-Sanchez *et al.*, 2007; Cornick & VuKhac, 2008). In particular, there is much evidence of the carriage of toxigenic *E. coli* O157 by small domestic ruminants (sheep and goats) (Chapman *et al.*, 1997; Heuvelink *et al.*, 1998; Meng *et al.*, 1998; Fegan & Desmarchelier, 1999; Ogden *et al.*, 2005). Furthermore, human cases and outbreaks of EHEC O157:H7 disease have often been linked to open farms or petting zoos where the organism has been isolated from animals including small ruminants (Shukla *et al.*, 1995; Chapman *et al.*, 2000; Pritchard *et al.*, 2000; Heuvelink *et al.*, 2002; Payne *et al.*, 2003; Stirling *et al.*, 2008). Other cases have been linked to small ruminant dairy products (Bielaszewska *et al.*, 1997; Steen *et al.*, 2001; McIntyre *et al.*, 2002; Espie *et al.*, 2006). With the increasing popularity of petting farms, the intensification of goat and sheep farming and the wider availability of sheep and goat products worldwide (including milk and unpasteurised cheeses), small ruminants are gaining attention as potentially significant reservoirs of *E. coli* O157:H7. The present review aims to provide insights into the work to date on the colonisation and persistence of *E. coli* O157:H7 in small domestic ruminants and the role that they may play as reservoirs of *E. coli* O157:H7 for human infection.

Sheep and goats as reservoirs for *E. coli* O157:H7

The prevalence of *E. coli* O157:H7 in small domestic ruminants is less well documented than in cattle and reports vary considerably. Battisti *et al*. (2006) reported a prevalence of 0.2% in lambs taken to slaughter in Italy. In the Netherlands the prevalence, using a sensitive immunomagnetic separation (IMS) culture technique on faeces at slaughter, varied from 3.8 to 4.1% of animals depending on age of animals sampled (Heuvelink *et al.*, 1998). A prevalence of 8.7% positive flocks was found in a Spanish study, also using IMS on rectal faeces samples, with around 7.3% of individuals within affected flocks found to be excreting *E. coli* O157:H7 (Oporto *et al.*, 2008). In England and Wales, using similar methodology, the individual prevalence varied between 1.7 and 2.2% (Chapman *et al.*, 1997; Paiba *et al.*, 2002) and more recently in Great Britain, toxigenic *E. coli* O157 was found in 0.7% of faeces samples at slaughter (Milnes *et al.*, 2008). In Scotland the IMS-determined prevalence of *E. coli* O157 among sheep pasture faeces samples was 6.5% (Ogden *et al.*, 2005). One study in the USA cultured faeces samples from 35 sheep on three occasions over six months and reported a peak prevalence, in June, of 31% *E. coli* O157:H7-positive animals (Kudva *et al.*, 1996).

In addition to the prevalence reports above, some studies have cited sheep products as important sources of *E. coli* O157:H7 (Rey *et al.*, 2006; Kalchayanand *et al.*, 2007). Furthermore, sheep have also been cited as reservoirs for a diverse number of non-O157 serogroups (including O26, O91, O115, O128 and O130) that encode a key colonisation factor in common with *E. coli* O157:H7, namely the potential to cause attaching-effacing (AE) lesions at the epithelial mucosa (Djordjevic *et al.*, 2001; Cookson *et al.*, 2002a; Blanco

et al., 2003; Aktan *et al.*, 2004; Cookson *et al.*, 2006; Kalchayanand *et al.*, 2007). Many of the above serogroups have been implicated in human disease. For example, a virulent O103:H25 EHEC strain has been traced directly to sheep products (Schimmer *et al.*, 2008). Non-O157 *E. coli* possessing key EHEC genes encoding intimin and Shiga-toxin are apparently more prevalent in sheep than is *E. coli* O157:H7 (Blanco *et al.*, 2003; Aktan *et al.*, 2004), although most of these non-O157 strains cannot be regarded as EHEC in the absence of evidence of their virulence in humans.

Attaching-effacing *E. coli* (AEEC) of various serogroups have been associated with enteric disease in goats (Tominaga *et al.*, 1989; Duhamel *et al.*, 1992; Drolet *et al.*, 1994; Barlow *et al.*, 2004; Wales *et al.*, 2005b). In addition, goats (as with other ruminants) can be sub-clinical carriers and excretors of *E. coli* O157:H7. Several recent reports have clearly identified (Bielaszewska *et al.*, 1997; Steen *et al.*, 2001; McIntyre *et al.*, 2002; Espie *et al.*, 2006) or implicated (Shukla *et al.*, 1995; Chapman *et al.*, 2000; Pritchard *et al.*, 2000; Payne *et al.*, 2003; Rey *et al.*, 2006) goats as sources of *E. coli* O157:H7 infection. Not only can goats be colonised with *E. coli* O157:H7, but their innately inquisitive behaviour means that they are much more likely than sheep to be in regular direct contact with humans, consequently increasing the risk of the direct faecal-oral transmission of zoonotic infection. There is little documented data on the prevalence of *E. coli* O157:H7 in goats, but studies have indicated a similar (Keen *et al.*, 2006; Fox *et al.*, 2007) or lower (Cortes *et al.*, 2005) prevalence to sheep.

E. coli O157:H7 colonisation factors

The ability to induce AE lesions has been studied extensively, and will be described more fully below, and it is this phenotype that seems to be the primary contributor to colonisation in all ruminant species including sheep and goats (Woodward *et al.*, 2003; La Ragione *et al.*, 2005b, 2006). For all AEEC, there are other factors such as flagella and fimbriae that also seem to play roles in modulating colonisation. The clinical outcome of infection in humans and animals seems to be directly related to the immune status of the host, and host susceptibility to the effects of colonisation and of Shiga-toxin (Stx), which is discussed below.

E. coli O157:H7 is able to induce AE lesions in the alimentary tract of humans and animals and isolates are typically positive for one or more of the Stx subtypes, and therefore are classified as EHEC. AEEC that do not elaborate Shiga toxins but which are associated with diarrhoeal disease are generally referred to as enteropathogenic *E. coli* (EPEC).

The attaching-effacing (AE) lesion

The AE lesion (Fig. 1) is characterised by intimate adherence between the bacterium and the host epithelial cell membrane, with an intervening gap of about 10 nm, plus effacement of enterocyte microvilli. Beneath the adherent bacterium a cytoskeletal rearrangement, including the accumulation of filamentous actin (F-actin), is seen. The bacteria often sit upon a pedestal-like structure, which can extend up to 10 µm away from the epithelial cell surface (Kaper *et al.*, 1998). The lesion is typically described as forming in three stages: initial non-intimate adhesion (mediated by elements that appear to vary between bacterial strains and AEEC types) is followed by signal transduction (leading to cytoskeletal reorganisation and microvillus effacement) and finally intimate attachment. Initial adhesion

in is poorly-understood for *E. coli* O157:H7: it may involve fimbrial organelles *in vivo*, as discussed later, but these have not been consistently identified or characterised, unlike the bundle-forming pili (BFP) that mediate initial attachment of typical EPEC. The EspA filaments of the secretion apparatus (discussed below) appear to have a non-intimate adhesin role.

Signal transduction and cytoskeletal reorganisation is effected using a type-III secretion apparatus, the elements of which are chromosomally-encoded in the Locus of Enterocyte Effacement (LEE) pathogenicity island. Esp ('EPEC-secreted proteins') A, B and D are thought to create a bi-functional adhesin and pore-forming channel, through which proteins, including the translocated intimin receptor Tir, are translocated into a host enterocyte. Tir ultimately localises to the host cell membrane, where it functions as a receptor for a bacterial surface adhesin (intimin) in the formation of an intimate attachment. In AE lesions formed by *E. coli* O157:H7, the recruitment of host cytoskeletal elements (F-actin and alpha-actinin) appears to be dependent on Tir cytoskeleton coupling protein (TccP), another effector protein that is translocated into host cells (Campellone *et al.*, 2004a, b; Garmendia *et al.*, 2004; Allen-Vercoe *et al.*, 2006). The role(s) of the cytoskeletal rearrangements remain speculative, but firm anchorage of the bacteria to the host cells is an obvious possibility. Although EHEC and EPEC AE lesions are morphologically very similar, significant differences in the respective mechanisms of formation are emerging, with EPEC Tir appearing to engage host cell elements without requiring TccP (Goosney *et al.*, 2000; Gruenheid *et al.*, 2001; Campellone *et al.*, 2004b; Allen-Vercoe *et al.*, 2006), although some EPEC do encode a TccP variant (Ooka *et al.*, 2007). Fuller descriptions of the process have been given elsewhere, for example by Wales *et al.* (2005a).

The first illustration of AE lesions in small ruminants was in sheep, and the lesions were observed in the small and large intestines of experimental lambs (Angus *et al.*, 1982). Thereafter, two natural cases with AE lesions were reported in diseased neonatal lambs (Janke *et al.*, 1989). AE lesions produced by untyped naturally-acquired bacteria were also observed on the ileal and large intestinal mucosa of symptomless neonatal lambs (Wales *et al.*, 2005b).

The Locus of Enterocyte Effacement (LEE)

A number of enteric pathogens, including EPEC, EHEC, *Citrobacter rodentium*, *Hafnia alvei* and rabbit EPEC (RDEC) may encode the LEE and so have the ability to induce AE lesions in the host intestinal tract (Agin *et al.*, 1996). Intimin is encoded by *eae* in the LEE of *E. coli* O157:H7; it is essential for AE lesion formation and has been cited as one bacterial factor for colonisation of the gastrointestinal tract (Donnenberg *et al.*, 1993; Dean-Nystrom *et al.*, 1999; Woodward *et al.*, 2003). Experimental studies have used various *E. coli* O157:H7 strains in oral inoculation studies in conventionally reared sheep to define colonisation and persistence patterns. Studies by Cornick *et al.* (2002) and Woodward *et al*. (2003) demonstrated that intimin contributes significantly to the colonisation and persistence of *E. coli* O157:H7 in sheep. In both studies intimin-deficient isogenic mutants showed reduced numbers excreted: around 1 log unit difference at 3 days post-inoculation and in excess of 2 log units difference by 15 days. Dean-Nystrom et al. (1999) reported that weaned calves inoculated with wild-type *E. coli* O157:H7 had a considerably greater density of the organism in the large intestine than did calves inoculated with an isogenic intimin-deficient mutant ($10^{6.6}$ versus $<10^3$ cfu/g). Cornick *et al.* (2002) showed that an intimin-deficient *E. coli* O157:H7 mutant was excreted in lower numbers and for a shorter period from inoculated yearling cattle and young adult sheep than was its co-inoculated wild-type

parent strain: there was a >1 log unit difference in numbers excreted up to 15 days post-inoculation, and by 30 days most animals had stopped excreting mutants but were still excreting the wild-type. However, similar studies conducted in goats (La Ragione *et al.*, 2005b) indicated a lesser role for intimin in colonisation and persistence, as an isogenic *eae* mutant was not attenuated for persistence over a period of 29 days (no significant difference in excreted numbers from 14 weaned kids inoculated with either wild-type or intimin-deficient *E. coli* O157:H7).

Tir, the LEE-encoded intimin receptor, was examined *in vivo* by Vlisidou *et al.* (2006), who concluded that it was essential for the colonisation of both lambs and calves, and that colonisation is mediated by Tir serving as a primary receptor for intimin. This report also concluded that *tir* mutants were more attenuated than *eae* (intimin) mutants, for example in lambs the *tir* mutant was undetectable by 3 days post-inoculation whereas the *eae* mutant persisted to day 16, implying that Tir may facilitate intestinal colonisation in other ways also. Stevens *et al.* (2004) has also reported a significant reduction in the colonising ability of a *tir* mutant in calves. Recent studies by Bretschneider et al. (2007) have described a similar reduced colonisation phenotype for Tir-deficient mutants in yearling beef cattle, although co-modulation of flagellin expression in these particular mutants complicates interpretation in this case.

The role of translocator and effector proteins

Numerous secreted proteins have been described in AEEC including in *E. coli* O157:H7. The LEE encodes the translocators EspA, EspB and EspD, plus characterised or putative effectors EspF, EspG, EspH, EspZ, Tir and Map. Certain non-LEE encoded proteins are also secreted by the type-III apparatus, including Cif (cycle-inhibiting factor), TccP, EspJ, EspG2 and Nle (non-LEE-encoded) proteins A, C and D. Deletion of the *LEE4* operon, abolishing or severely curtailing the type-III apparatus and delivery of proteins secreted by this route, prevented the colonisation of calves by *E. coli* O157:H7 (Naylor *et al.*, 2005).

In addition to Tir (discussed above), EspA and TccP have been investigated individually in *E. coli* O157:H7. Experimental mutation of *espA* strongly reduced the colonisation of calves (Dziva *et al.*, 2007), but *tccP* mutation did not impair the colonisation of calves or lambs (Vlisidou *et al.*, 2006). The *tccP* mutant was unable to nucleate F-actin on human HeLa cells *in vitro*, although it adhered well. However, *in vivo* in calves it was able to induce AE lesions with typical pedestals, suggesting that EHEC pedestal formation *in vivo* may differ from that *in vitro*: possible reasons include host cell differences or effector molecules being available from the surrounding flora *in vivo*. Using an alternative approach, immunisation of cattle by injection of an adjuvanted mix of Esp proteins, with or without Tir, significantly reduced the shedding of *E. coli* O157:H7 amongst orally dosed calves and naturally-exposed adults (Potter *et al.*, 2004).

EspJ inhibits macrophage phagocytosis and is not required for AE lesion formation (Marchès *et al.*, 2008). Dahan *et al*. (2005) showed that *espJ* mutation actually enhanced the persistence of both a non-toxigenic *E. coli* O157:H7 strain and a *C. rodentium* strain in conventionally reared six-week old lambs and in mice, respectively. This was manifested as three of five *espJ* mutant-inoculated lambs versus one of five wild-type-inoculated lambs excreting *E. coli* O157:H7 at nine days post-inoculation, and four of five *espJ* mutant-inoculated mice versus none of five wild-type-inoculated mice having inoculum in the caecum at 20 days post-inoculation. Thus, EspJ appears to have properties that might enhance virulence (by inhibiting an aspect of the host immune response) but, on the limited evidence available, EspJ may also limit persistence.

Cif (cycle-inhibiting factor) is encoded on a lambdoid phage and many EHEC and EPEC strains carry functional or mutated forms of *cif*. It triggers an irreversible cytopathic effect in HeLa cells, which is characterised by the progressive recruitment of focal adhesions, the assembly of stress fibres and arrest of the cell cycle (Nougayrede *et al.*, 2001; Marchès *et al.*, 2003). It has been hypothesised (Marchès *et al.*, 2003) that Cif-dependent arrest of the host cell cycle in a population with rapid turnover, such as enterocytes, may aid colonisation and persistence, although studies to examine this have not yet been reported.

EspI/NleA is encoded within prophage CP933P on the *E. coli* O157:H7 genome; it localises to the host cell Golgi apparatus and inhibits secretion mechanisms (Gruenheid *et al.*, 2004; Kim *et al.*, 2007). The *nleA* (*espI*) gene has been found widely in EHEC (O157 and non-O157) and EPEC, and appears to be associated with virulence in humans (Mundy *et al.*, 2004a; Creuzburg & Schmidt, 2007). In *C. rodentium*, it is required for full virulence in susceptible mice and for the normal colonisation of resistant mice (Gruenheid *et al.*, 2004; Mundy *et al.*, 2004b). NleC and NleD were examined by Marchès *et al*. (2005): a wild-type *E. coli* O157:H7 strain and isogenic mutants in *nleC* and *nleD* were separately orally inoculated into groups of lambs, and all strains were excreted at the same density in faeces for the following seven days. It was concluded that neither protein was required for the colonisation and/or persistence of *E. coli* O157:H7 in a six-week-old conventional lamb model.

The products of the plasmid-borne *toxB* and the truncated chromosomal *efa-1'* genes appear to enhance LEE-dependent secretion in *E. coli* O157:H7 and adherence to host cells *in vitro*, without having obvious effects on its persistence when tested by monitoring of the levels of excretion following oral inoculation of parent and isogenic mutant strains into sheep and calves (Stevens *et al.*, 2004).

Thus, amongst the substantial array of *E. coli* O157:H7 translocator or type-III apparatus–secreted proteins, only Tir and EspA have been shown individually to have major roles in colonisation and persistence in ruminants, and of these only Tir has been examined in sheep. A few others have been found not to enhance colonisation, but there is very limited data available on many of the proteins, most of which have not been examined until very recently. Therefore their potential contributions to differences between *E. coli* O157:H7 colonisation of different hosts, or to tissue tropisms, remain unknown. For those which are apparently not important in the context of ruminant colonisation and persistence, it may be that the bacterium is able to compensate for the lack of a single effector and/or translocator protein or is able to harness available protein from AEEC in the natural flora, for example at sites of pre-existing AE lesions. In addition, it is possible that multiple knockout mutants are required to see significant effects, as many of these translocator and effector proteins probably work in a synergistic manner. The issue of redundancy needs also to be considered for some effectors as, for example, EspG2 is functionally similar to the LEE-encoded EspG but is encoded elsewhere on the EPEC chromosome (Elliott *et al.*, 2001; Shaw *et al.*, 2005).

The role of toxins in colonisation and persistence

One of the major virulence factors of *E. coli* O157:H7 are the Shiga- (Vero-) toxins, which occur as two major subtypes (Stx1 and Stx2) and are encoded by lysogenic bacteriophages. The preferred receptor for the toxin is globotriaosylceramide (Gb_3). In humans, intestinal elaboration of Stx is associated with a microvascular angiopathy in the intestine and other critical organs, including kidney and central nervous system (CNS),

which accounts for both the typical bloody diarrhoea and the frequently severe systemic manifestations of EHEC infection (Richardson *et al.*, 1988; Baker *et al.*, 2007). Piglets express Gb_3 receptors in renal and CNS vasculature and show corresponding angiopathies on exposure to Stx (Gunzer *et al.*, 2002), but cattle lack vascular Gb_3 receptors in those organs principally affected in humans (Pruimboom-Brees *et al.*, 2000; Hoey *et al.*, 2002), which may explain why ruminants are symptomless carriers of *E. coli* O157:H7.

Studies to date have shown that non-Shigatoxigenic *E. coli* O157:H7 can colonise cattle, sheep, goats and chickens with no clinical manifestations, even though AE lesions may form in the gastro-intestinal (GI) tract (Woodward *et al.*, 2003; Best *et al.*, 2005; La Ragione *et al.*, 2005b, 2006). It has been postulated that Stx may play a role in the ability of *E. coli* O157:H7 to persist in ruminants, and *in vitro* studies have pointed to this, showing an inhibitory effect of Stx1 on bovine lymphocytes (Menge *et al.*, 2003). However, recent studies by Cornick *et al*. (2007) have indicated that non-toxigenic *E. coli* O157:H7 strains, created from a wild-type either by deletion mutation of *stx2* or by curing the parent of its *stx* phage, compete effectively with their toxigenic progenitor strain in the colonisation of sheep. Stevens et al. (2002) concluded that the elaboration of Stx1 by EHEC O103:H2 did not influence enteric inflammatory responses (fluid accumulation and recruitment of radio-labeled neutrophils), in a bovine ligated ileal loop model. Another toxin associated with *E. coli* O157:H7 is enterohaemolysin, but this has not been investigated in relation to persistence of the bacterium.

The role of fimbriae and lipopolysaccharide in colonisation and persistence

Fimbriae have been shown to be important in the pathogenesis of a number of *E. coli* pathotypes (La Ragione *et al.*, 2000; Lane & Mobley, 2007) and recent studies have revealed the presence of at least 16 fimbrial gene clusters in *E. coli* O157:H7. Many of these fimbriae do not appear to be expressed *in vitro*, and in particular type 1 fimbrial expression is blocked by a 16bp deletion in the '*fim* switch' regulator (Iida *et al.*, 2001; Roe *et al.*, 2001).

Long polar fimbriae (LPF), originally found to be important for the pathogenesis of *Salmonella* Typhimurium (Bäumler *et al.*, 1996), represent one potential adherence determinant in *E. coli* O157:H7, which contains two non-identical *lpf* loci homologous to *lpf* of *S*. Typhimurium (Perna *et al.*, 2001). Torres *et al*. (2002) showed that the *lpf1* operon increases adherence of *E. coli* K12 to cultured epithelial cells *in vitro* and was associated with long peritrichous fimbriae. Furthermore, mutation of both these *lpf* loci together in *E. coli* O157:H7 reduced the numbers recovered from orally inoculated pigs and sheep (Jordan *et al.*, 2004), an effect most pronounced in the first two weeks of the eight-week study. Mutation of either *lpf* locus altered the tropism of *E. coli* O157:H7 adhesion (with AE lesion formation) on human intestinal explants, paradoxically expanding the range of adhesion sites from ileal follicle-associated epithelium (wild-type tropism) to include other small intestinal epithelium (Fitzhenry *et al.*, 2006). More recently, Torres *et al*. (2007) showed that mutation of both *lpf* loci (but not either singly) reduced the ability of *E. coli* O157:H7 to persist in the intestine of orally infected conventionally reared 6-week-old lambs. Interestingly, the same study showed *lpf* mutants to be better colonisers of intestinal mucosal explants than wild-type strains, suggesting that the LPF role in persistence may not involve initial adherence, contrary to what might be assumed.

Under certain culture conditions type IV pili (Xicohtencati-Cortes *et al.*, 2007) and 'F9' fimbriae (Low *et al.*, 2006) can be observed and extracted from *E. coli* O157:H7 strains. The type IV pili appear to assist adhesion of *E. coli* O157:H7 to human, cattle and pig gut

explants, but *in vivo* studies are currently lacking. Fimbrial mutant studies in calves have not identified a clear role in colonisation for the F9 fimbriae. Sorbitol-fermenting EHEC O157:H- strains have plasmid-encoded fimbriae that are only expressed in anaerobic conditions, and which enhance adhesion to human intestinal epithelial cell lines (Müsken *et al.*, 2008). For other AEEC subtypes such as EPEC, the role of fimbriae is clearer. In particular, bundle forming pili (BFP) have been shown to be important in initial attachment (Knutton *et al.*, 1987).

In vivo studies have concluded that mutants lacking O157 lipopolysaccharide (*gal* knockouts) were uniformly attenuated in an infant rabbit intestinal colonisation model, as might be anticipated (Ho & Waldor, 2007).

The role of flagella in colonisation and persistence

Motility processes in Gram negative bacteria are complex and require the co-ordinated transcription of more than 40 genes in 14 operons (Iino *et al.*, 1988; Liu & Matsumura, 1994). Flagellum-driven motility has been shown to be associated with adherence to epithelial cells by many diverse *E. coli* pathotypes and other pathogenic bacteria (Feldman *et al.*, 1998; Allen-Vercoe & Woodward, 1999; La Ragione *et al.*, 2000; Tasteyre *et al.*, 2001; Giron *et al.*, 2002; Inglis *et al.*, 2003; Dons *et al.*, 2004; Kirov *et al.*, 2004; Wright *et al.*, 2005). Flagella, including H7, have been recognised as potent inflammatory ligands, evoking inflammatory responses through engagement with a number of receptors (Berin *et al.*, 2002; Zhou *et al.*, 2003). Studies have shown that quorum sensing pathways positively regulate the expression of *E. coli* O157:H7 flagella (Sperandio *et al.*, 2001, 2002), in addition to regulating other virulence factors including the type III secretion system (Sperandio *et al.*, 1999). However, sorbitol-fermenting *E. coli* O157:H- are almost always non-motile, but are still able to cause clinical disease in humans (Monday *et al.*, 2004), indicating that flagella are not essential for EHEC virulence in the human host. In experimentally infected pigs, a flagella-deficient *E. coli* O157 mutant persisted as well as its isogenic progenitor O157:H7 strain (Best *et al.*, 2006) but, by contrast, in a surrogate SPF chick model of EHEC colonisation (Best *et al.*, 2005) aflagellate *E. coli* O157 mutants were significantly less persistent than their wild-type progenitor strain. Recent studies conducted by La Ragione *et al*. (2005a) demonstrated, using a flagellin gene (*fliC*) knockout mutant, that flagella did not contribute to the long-term persistence of *E. coli* O157:H7 in goats, and indeed isogenic *fliC* mutants showed a tendency to be excreted for longer than intact parent strains. Interestingly, cattle studies have shown that an intact flagellar regulatory gene (*flh*C) contributes to effective host colonisation but, similar to ovine studies, the *fliC* gene responsible for filament formation does not appear to be important in this context (Dobbin *et al.*, 2006). In addition, the same study reports that aflagellate (Δ*fli*C) *E. coli* O157:H7 are able to survive passage through the bovine GI tract better than flagellated strains. Data reported by Bretschneider *et al*. (2007) apparently show that *E. coli* O157 deficient in the expression of flagella were attenuated in their capabilities of colonizing experimentally infected beef cattle, although the aflagellate state in this case was an uncontrolled and undefined by-product of genetic manipulations of *tir*.

Erdem *et al.* (2007) recently demonstrated that flagella from EHEC and EPEC may mediate adherence to mucus in the intestinal tract, whereas McNeilly *et al*. (2007) showed that mucosal samples from *E. coli* O157:H7-exposed cattle contained antibodies to H7. Therefore, potential benefits to *E. coli* O157:H7 colonisation by flagellar motility or adhesion might be counteracted in the longer term by mucosal immune responses elicited by flagellin.

E. coli O157:H7 and tissue tropisms

Tissue tropism has been identified as important in the pathogenesis of *E. coli* O157:H7. Naylor et al (2003) reported that nine of 10 experimentally-inoculated weaned calves excreting *E. coli* O157:H7 for a minimum of 2 weeks, plus one naturally-infected steer, showed a significantly raised density of *E. coli* O157 in the region of the recto-anal junction (RAJ). Evidence of adherent *E. coli* O157 bacteria in this area was also presented. A Shiga toxin-negative *E. coli* O157:H7 strain was subsequently shown to demonstrate LEE-dependent persistence and to form AE lesions at the RAJ in experimentally inoculated calves (Naylor *et al.*, 2005). The same group (Low *et al.*, 2005) showed, in an abattoir survey, a similar pattern of increasing density of *E. coli* O157:H7 with increasing proximity to the RAJ on the bovine rectal mucosa, and also an association between *E. coli* O157 density at the RAJ and in the faeces. A different group of workers (Rice *et al.*, 2003) provided corroborative evidence of the importance of the RAJ in bovine colonisation by showing that swabs of the RAJ in experimentally and naturally colonised cattle provided more sensitive detection of *E. coli* O157:H7 than did faecal samples. The same group (Sheng *et al.*, 2004) showed that rectal inoculation of calves with a sponge soaked in $\geq 10^7$ cfu *E. coli* O157:H7 resulted in persistent excretion, with colonisation that could only be detected at the rectum. By contrast, an oral inoculum of 10^{10} cfu of the same strain achieved a pattern of persistent excretion over eight weeks that was similar to that seen with a rectal inoculum of 10^7 cfu and significantly less, in terms of the percentage of animals excreting, than that seen with a rectal inoculum of 10^{10} cfu. Recent studies by Bonardi *et al*. (2007) have suggested that *E. coli* O157 may actually enter into bovine lymphatic tissues such as the mesenteric lymph nodes and tonsils.

The studies conducted by La Ragione *et al*. (2005b, 2006) do not suggest that the RAJ is a site of primary importance leading to persistent colonisation and shedding of *E. coli* O157:H7 in sheep and goats . These studies concluded that *E. coli* O157:H7 colonised the distal intestine including the caecum, colon and rectum, suggesting that this area of the GI tract is the preferred site of colonisation in these species. Detection of AE lesions was rare with only small and sparse lesions seen, even though the challenge strain was able to induce diffuse lesions *in vitro* to cultured epithelial cells and to adhere efficiently to ovine *in vitro* organ culture (Dibb-Fuller *et al.*, 2001; Torres *et al.*, 2007). The same studies (La Ragione *et al.*, 2005b, 2006) showed no specific tropism of *E. coli* O157:H7 towards lymphoid tissue on microscopic examination, albeit that pathological examinations were only performed up to four days post-inoculation. Cookson *et al.* (2002b) showed a positive correlation for *E. coli* O157:H7 between the numbers consistently excreted and the extent of its distribution in the GI tract over a timescale of up to 3 weeks after oral inoculation. More recent studies in sheep (Woodward *et al.*, 2003) include the observation that animals colonised beyond approximately 14 days post-infection (and therefore considered to be persistent shedders) had *E. coli* O157 organisms throughout the entire GI tract, rather than just the large intestine.

Collectively, these findings suggest that in small ruminants colonisation of the GI tract may occur throughout the distal region and be diffuse, not preferentially targeted to the RAJ as in cattle. However, to confound this general conclusion, Grauke *et al*. (2002) found that persistently-excreting experimentally infected sheep showed very rare evidence of *E. coli* O157:H7 in the intestine to the level of the descending colon. Moreover, either rectal or oral inoculation with *E. coli* O157:H7 leads to similar faecal shedding in lambs (Best A, Clifford D, Crudgington B, Cooley WA, Nunez A, Carter B, Weyer U, Woodward MJ & La

Ragione RM, unpublished data[1]) , and where rectally inoculated lambs were positive for *E. coli* O157:H7 at necropsy, the majority of bacteria were associated with the recto-anal mucosa. This may not be surprising, given that the inocula were directed to the rectum and previous data indicated that all of the distal GI tract is susceptible to colonisation. To suggest that this site may indeed be a site of preferential colonisation is perhaps erroneous and an artefact of the mode of administration. Additionally, further analysis by confocal microscopy revealed that only a small subset of rectally inoculated lambs had large, densely-packed *E. coli* O157:H7 micro-colonies identified by specific staining. What these data do confirm is that *E. coli* O157:H7 can effectively colonise the very distal region of the GI tract. Interestingly, in rectally inoculated lambs, *E. coli* O157:H7 was not as frequently recovered from other GI tract tissues when compared with orally inoculated lambs. Given the route of administration, this also may not be surprising.

Oral inoculation studies with a non-toxigenic ovine-derived *E. coli* O26:K60 strain (Aktan *et al.*, 2007) showed that the challenge organism could be recovered from all sites in the GI tract during the high excretion phase, but from just the distal small intestine during long-term persistence, 38 days after inoculation. This illustrates that tissue tropisms probably vary amongst differing persistent AEEC, including those in common EHEC and EPEC serogroups. Indeed, in the several small ruminant *E. coli* O157:H7 infection studies reported, of particular interest is the frequent occurrence of AE lesions caused by non-O157 bacteria, some of which have been identified as belonging to O26 and O115 serogroups (Cookson *et al.*, 2002a; Wales *et al.*, 2005b, c). Sheep appear to be reservoirs for a diverse range of AEEC with varying serotypes, virulence profiles and intimin types (Aktan *et al.*, 2004; Cookson *et al.*, 2007), and it may be hypothesised that different intimin types facilitate tropism to different specific sites in the GI tract, as described for cattle and humans (Fitzhenry *et al.*, 2002; Mundy *et al.*, 2007), or perhaps that some intimin subtypes play no role in the colonisation of AEEC in sheep.

Effects of host factors on *E. coli* O157:H7 colonisation and persistence

General health, hormonal and immune status

Recently, interest has developed in host factors relating to the virulence and persistence of EHEC and EPEC, particularly with the advent of genome sequences and the widespread use of microbial and host arrays. It remains unclear at present how stress and hormonal stimulation may influence colonisation in any species. One reasonably well-described mechanism by which host stress can alter the behaviour of *E. coli* O157:H7 is the influence of the catecholamine hormones epinephrine and norepinephrine on the ‘quorum-sensing’ bacterial system, by which virulence mechanisms can be upregulated (Sperandio *et al.*, 2003) and enteropathic effects such as adhesion and inflammation can be enhanced (Vlisidou *et al.*, 2004).

Several researchers have considered the immune status of the host. It is well-established that colostrum deprivation is a risk factor for increased susceptibility to gastrointestinal pathogens (Kelleher & Lonnerdal, 2001) and that colostrum is protective against pathogenic *E. coli* (Logan *et al.*, 1974; Altmann & Mukkur, 1983). It has also been demonstrated in neonatal calves that colostrum is highly protective against *E. coli* O157:H7 challenge and that AE lesions are readily induced in the distal GI tract if colostrum is

[1] Intermittent *Escherichia coli* O157:H7 colonisation at the terminal rectum mucosa of conventionally-reared lambs. Paper submitted to Veterinary Research.

withheld (Dean-Nystrom *et al.*, 1997; Rugbjerg *et al.*, 2003). La Ragione *et al*. (2005a, 2006) showed that colostrum deprivation increased susceptibility to *E. coli* O157:H7 colonisation in young lambs and that colostrum was protective for sucking goat kids in the face of a modest challenge via the nanny, with no evidence that intra-mammary infection was significant.

The general health status of the ovine or caprine host may have a major influence on persistence and high level shedding of *E. coli* O157:H7. However, there is at present a lack of information in this area, including the influence of concurrent infection status, although some work (reported below) reveals the effect of cryptosporidiosis. Survey data looking at a range of infective agents may be a useful approach in the future. There is also a lack of hard data on transmission routes in farmed animal species, although experimentally and epidemiologically the concept of 'supershedders' being important in the maintenance of herd status has been developed in relation to *E. coli* O157:H7 in cattle (Cobbold *et al.*, 2007).

The possible role of concurrent colonisation by other attaching-effacing *E. coli* or pathogens such as *Cryptosporidium*

Competition between bacteria and the dominance of certain *E. coli* serotypes or strains is suggested to be one explanation for the shedding of one predominant *E. coli* strain over time (Midgley *et al.*, 1999). La Ragione *et al*. (2004) reported on the interactions between *E. coli* O26:K60 and *E. coli* O157:H7 in tissue culture adherence assays, which showed that pre-incubation of tissue culture cells with either strain reduced significantly the extent of adherence of the strain that was applied second. Aktan *et al.* (2007) reported that atypical EPEC O26:K60 colonised six-week-old conventionally reared lambs after oral inoculation, with persistent shedding for well over a month and with the induction of AE lesions that were small and sparse in the distal GI tract. In the field, more than one group has shown *E. coli* serogroup O26 to be prevalent in ruminant animals at slaughter when the prevalence of *E. coli* O157:H7 in the same study animals was very low (Aktan *et al.*, 2004; Fukushima & Seki, 2004).

Taken together, these data suggest that the colonisation and shedding of lambs by *E. coli* O157:H7 may be altered, and possibly reduced, if the lambs are previously colonised by EPEC O26:K60. However, further experimental studies (Aktan I, La Ragione RM & Woodward MJ, unpublished data) revealed that prior infection with *E. coli* O26:K60 did not alter the colonisation or persistence of subsequent *E. coli* O157:H7 infections. Interestingly, when the order of administration was reversed, prior experimental colonisation of conventionally reared lambs by *E. coli* O157:H7 did significantly suppress *E. coli* O26:K60 colonisation and persistence, an interaction that requires further investigation.

Cryptosporidium parvum has been shown to be a common agent associated with diarrhoeal disease in young calves and lambs in Europe, often as part of mixed enteric infections (Munoz *et al.*, 1996; de la Fuente *et al.*, 1999). La Ragione *et al*. (2005b) showed that an experimental conventionally-reared goat kid shedding especially high numbers of *E. coli* O157:H7 was also heavily infected with *Cryptosporidium*. Further studies (La Ragione *et al.*, 2006) revealed that lambs experimentally pre-inoculated with *C. parvum* prior to challenge with *E. coli* O157:H7 shed very high numbers of the *E. coli* challenge strain and developed extensive, multifocal AE lesions in the caecum, colon, rectum and at the RAJ (Fig. 2). Lesions were confirmed by immunohistochemistry to be associated with *E. coli* O157.

Thus, concurrent infection with an unrelated pathogen may enhance colonisation by *E. coli* O157:H7 and its excretion. Indeed, it is also possible that viral infections may play a

role in predisposing ruminants to AEEC, including *E. coli* O157:H7. There have been several reports where rotavirus has been isolated from animals presenting with clinical signs associated with *E. coli* O26 (Acres *et al.*, 1977; Janke *et al.*, 1990). In addition, it may be hypothesised that immunosuppressive viruses, for example bovine immunodeficiency virus or bovine viral diarrhoea virus in cattle, and border disease virus or maedi-visna virus in sheep, could influence AEEC colonisation and persistence.

Summary and concluding remarks

Numerous putative colonisation factors have been identified and characterised in *E. coli* O157:H7, and recent publication of AEEC genomes and comparative genomics (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Zhang *et al.*, 2007) has enabled the analysis of the genome for specific genes that may contribute to persistent colonisation of ruminants.

Studies to date include the characterisation of defined targeted mutants constructed in *eae, tir, efa*/*toxB, fliC, lpf, nleD, tccP,* made primarily in the non-Shigatoxigenic *E. coli* O157:H7 strain NCTC12900. The data compiled to date points to a role for intimin and Tir in sheep and cattle (Woodward *et al.*, 2003; Vlisidou *et al.*, 2006) although, interestingly, the colonisation of goats seems less dependent upon intimin. Indeed, the studies by La Ragione *et al*. (2005b, 2006) and Woodward *et al*. (2003) suggest that the long-term persistence of *E. coli* O157:H7 in small ruminants may be significantly affected by bacterial factors in addition to intimin and Tir, as well as by host factors such as adequacy of colostrum intake in the young or prior colonisation by other pathogens, or possibly other elements of the commensal flora. Other than certain LEE-encoded factors (intimin, Tir, EspA), few of the other candidate bacterial factors thus far examined have a significant effect on colonisation and/or persistence when tested in experimental ruminant (sheep and cattle) models of colonisation (Woodward *et al.*, 2003; Stevens *et al.*, 2004; Marchès *et al.*, 2005; Vlisidou *et al.*, 2006; Torres *et al.*, 2007).

It is clear that *E. coli* O157:H7 is prevalent in small ruminants (sheep and goats) and that groups of these animals may act as effective reservoirs for this important zoonotic pathogen. Much has been revealed about the pathobiology of *E. coli* O157:H7 in cattle, including a LEE-dependent tropism for lymphoid tissue at the terminal rectum. This precise tropism has not been observed in small ruminants, but it is evident that colonisation and persistence of sheep and goats is influenced by the LEE-encoded secretion system. Localisation of *E. coli* O157:H7 has been observed at the ovine recto-anal mucosa, albeit intermittently. In both cattle and small ruminants, certain other secreted proteins, surface appendages, age and stress effects are suspected to influence persistence, based upon *in vitro* studies and limited numbers of *in vivo* studies in laboratory and ruminant species. There is also, for small ruminants, some recent data showing that colonisation is enhanced in young animals with compromised passive immunity or those experiencing co-infection with an unrelated pathogen. What is less well understood for all ruminant reservoirs is the relationship between *E. coli* O157:H7 and the normal gastrointestinal flora, and how this might be modulated for the development of control strategies.

Research in the field of ruminant persistence of EHEC has so far identified some important elements that may be susceptible to practical interventions for control of *E. coli* O157:H7. A more comprehensive understanding of the LEE and non-LEE encoded factors that promote effective colonisation in the distal ovine GI tract is required in order to develop and assess the appropriateness of intervention strategies for the control of *E. coli* O157:H7 in the field. However, unless the same experiments are performed with the same strains and mutants in different animal species, we will struggle to arrive at truly solid conclusions

about differing behaviours that are host-specific. It seems likely that these insights will be needed to provide more comprehensive control of these subtle pathogens.

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Figure legends

Fig. 1. Attaching-effacing (AE) lesion. Transmission electron micrograph. **B**: Attached *E. coli* bacterium. **P**: host cell pedestal. **M**: host cell microvillus.

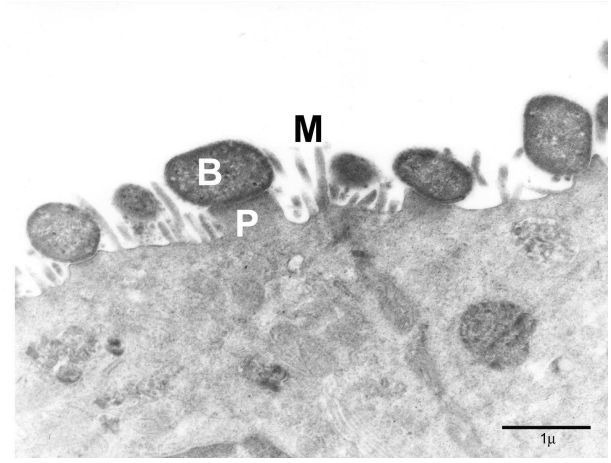

Fig. 2. Attaching-effacing lesions formed by *E. coli* O157:H7 at the recto-anal junction of a lamb co-infected with *Cryptosporidium parvum*. Transmission electron micrograph. **CP**: *C. parvum* cell attached to host mucosa. **EC**: *E. coli* O157:H7 intimately attached to host mucosa. **RM**: host rectal mucosal cell.