**REVIEW
ARTICLE****Options for the control of enterohaemorrhagic
Escherichia coli in ruminants**

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Overview

Enterohaemorrhagic *Escherichia coli* (EHEC) comprise an important group of zoonotic enteric pathogens. In humans, some EHEC infections result in bloody or non-bloody diarrhoea, which may be complicated by haemorrhagic colitis and severe renal and neurological sequelae, including haemolytic uraemic syndrome (HUS). Ruminants are an important reservoir of EHEC and human infections are frequently associated with direct or indirect contact with ruminant faeces. Strategies to reduce the prevalence of EHEC in ruminants should lower the incidence of human infection. However, little is currently known about the mechanisms of intestinal colonization of ruminants by EHEC and no effective vaccines have yet been developed. Here we review current knowledge on the host responses to EHEC infection and the factors mediating EHEC-intestinal interactions and survival in cattle and sheep. Perspectives for the use of vaccines, probiotics and other measures to control EHEC in farm animals are discussed.

Epidemiology of EHEC

EHEC are an emerging group of enteric pathogens of animals and humans (Nataro & Kaper, 1998). Human infections caused by EHEC have been reported with increasing frequency over the past 15 years in many countries and may involve severe enterocolitis and systemic sequelae (Slutsker *et al.*, 1997; Anon, 1998; Paton & Paton, 1998). The leading antecedent to EHEC infection is direct or indirect contact with ruminant faeces (Locking *et al.*, 2001; O'Brien *et al.*, 2001). Outbreaks are commonly attributed to the consumption of contaminated meat, milk and dairy products, in particular those derived from cattle (Griffin, 1995; Armstrong *et al.*, 1996); however, outbreaks have also resulted from the consumption of water, unpasteurized apple drinks and vegetables contaminated with

ruminant faeces (Olson *et al.*, 2002; Cody *et al.*, 1999; Hilborn *et al.*, 1999). Direct contact with ruminant faeces on farms and on contaminated pastures has also resulted in outbreaks of EHEC infection (O'Brien *et al.*, 2001; Ogden *et al.*, 2002). Farm animals are widely regarded to be the principal reservoir of EHEC, but the bacteria have been detected in a growing number of wild animals and birds (Mainil, 1999). Wild and domestic animals may act as vectors for transmission of EHEC to farm animal hosts and on rare occasions humans. Indeed rabbits were recently implicated in an outbreak of EHEC infection in visitors to a wildlife centre in Great Britain (Pritchard *et al.*, 2001).

The O-serotype predominantly associated with human infections in Great Britain is O157 and isolations have almost doubled in the last 6 years (Anon, 2000). Other EHEC serotypes (particularly O26, O111, O128 and O103) are emerging as a common cause of human disease in many countries and indeed may be more prominent than O157 (Bettelheim, 2000). It is presently unclear if the incidence of human infections caused by these serotypes correlates with the prevalence of the bacteria in farm animal hosts. In a recent survey of non-O157 Shiga-toxin-producing *E. coli* isolates from Scottish cattle, 8 of the 11 different O-serogroups represented have also been associated with bloody diarrhoea in humans and HUS (Jenkins *et al.*, 2002). Interestingly, O26 and O111 EHEC were not isolated in this study, although they have been isolated from healthy cattle in other studies (Kobayashi *et al.*, 2001). It remains unclear if all EHEC present in animal reservoirs present a risk to the human population. Further, it is not known why some EHEC serotypes (e.g. O157) can cause disease in humans but not in cattle. EHEC isolated from healthy cattle often possess the same combinations of putative virulence genes that are found in human isolates (Wieler *et al.*, 1996; Sandhu *et al.*, 1996; Kobayashi *et al.*, 2001; Jenkins *et al.*, 2002). However, differences in expression of these genes have been detected among human and bovine isolates *in vitro* and this may affect

the potential for zoonotic transmission (McNally *et al.*, 2001).

In a recent 12-month survey of cattle and sheep presented for slaughter at abattoirs in Great Britain, 4.7% of cattle and 1.7% of sheep contained *E. coli* O157 in the faeces (Paiba *et al.*, 2002). Further, it was reported that up to 44% of cattle herds in Great Britain have at least one animal shedding *E. coli* O157 (Paiba *et al.*, 2002). The *E. coli* O157 phage types most frequently isolated from cattle (2, 8 and 21/28) and sheep (4 and 32) were the same five types predominantly associated with illness in humans in Great Britain during the same period (Paiba *et al.*, 2002). The incidence of EHEC serotypes other than O157 that may pose a risk to humans was not determined in this study. In the United States, the prevalence data are even higher, with an estimated 28% of beef cattle shedding *E. coli* O157:H7 in the summer months (Gansheroff & O'Brien, 2000; Elder *et al.*, 2000).

The prevalence of EHEC is higher in younger animals and in animals subject to transit, feed changes and antimicrobial therapy, possibly due to disturbance in the resident intestinal microflora (Hancock *et al.*, 1998). The shedding patterns are characterized by sharp bursts of EHEC excretion in a high percentage of animals separated by longer periods of very low prevalence. *E. coli* O157:H7 is capable of efficient transmission between animals, even at very low doses (Besser *et al.*, 2001). To add to the problem, animals colonized by EHEC are hard to identify since *E. coli* O157:H7 infections in weaned calves and adult cattle and sheep are asymptomatic (Cray & Moon, 1995; Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1998a, 1999; Cornick *et al.*, 2000; Wray *et al.*, 2000). The infectious dose for *E. coli* O157:H7 in humans has been estimated to be less than 100 c.f.u. (Griffin, 1995). Calves and adult cattle experimentally infected with EHEC can excrete large numbers of EHEC for up to several weeks depending on the inoculation dose (Cray & Moon, 1995; Brown *et al.*, 1997; Sanderson *et al.*, 1999; Cornick *et al.*, 2000; Wray *et al.*, 2000). The magnitude and duration of excretion of EHEC by naturally infected animals is less well studied, therefore the risk to human health posed by ruminants colonized by EHEC is difficult to quantify. Some EHEC serotypes (e.g. O5, O26 and O118) are associated with enteric disease in farm animals and cause significant morbidity and mortality (Mainil, 1999; Bettelheim, 2000).

Host responses to EHEC infection

Infection of the gastrointestinal tract of adult cattle, weaned calves and 5-day-old gnotobiotic calves by EHEC serotype O157:H7 is asymptomatic (Cray & Moon, 1995; Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1999; Sanderson *et al.*, 1999; Woodward *et al.*, 1999; Wray *et al.*, 2000). Histological analysis of intestinal epithelia from calves and cattle infected with *E. coli* O157:H7 reveals intimate bacterial adherence in some but not all cases and a mild inflammatory response

characterized by diffuse infiltration of neutrophils into the lamina propria. Infection of neonatal colostrum-deprived calves with *E. coli* O157:H7 results in diarrhoea and colonic oedema (Dean-Nystrom *et al.*, 1997); however, such animals are atypically susceptible to infection. *E. coli* O157:H7 also persistently colonizes the intestines of neonatal lambs and sheep in the absence of clinical signs (Kudva *et al.*, 1995, 1997; Cornick *et al.*, 2000; Wales *et al.*, 2001a, b).

Natural and experimental infection of susceptible calves with bovine virulent EHEC serotypes frequently produces diarrhoea, villous atrophy, epithelial cell damage, infiltration of neutrophils into the lamina propria and intestinal lumen and the formation of a pseudomembrane containing blood, fibrin, cellular debris and neutrophils (Hall *et al.*, 1985; Chanter *et al.*, 1986; Pearson *et al.*, 1999; Mainil, 1999). Similar histopathological lesions occur in some cases of EHEC-associated haemorrhagic colitis in humans (Slutsker *et al.*, 1997).

Distal sites in the intestine such as the caecum, colon and rectum are believed to be the principal sites of *E. coli* O157:H7 colonization in ruminants (Cray & Moon, 1995; Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1998a, 1999; Wales *et al.*, 2001a, b; Grauke *et al.*, 2002). We and others have observed little, if any, direct interaction between *E. coli* O157:H7 and the epithelium of the lower gastrointestinal tract in experimentally infected conventional calves (M. Stevens, unpublished observations; Wray *et al.*, 2000). In contrast, extensive bacterial adherence to the colonic epithelium is seen in calves infected with EHEC serotype O5 (Hall *et al.*, 1985; Chanter *et al.*, 1986), O26 (Pearson *et al.*, 1999) and O111 (Stevens *et al.*, 2002b). Similar observations have been made in bovine ileal and colonic loops infected with O157 and non-O157 strains (Sandhu & Gyles, 2002). Thus *E. coli* O157 and non-O157 serotypes may use distinct mechanisms to colonize the ruminant host. Differences in tissue tropism may have implications for the control of EHEC colonization in cattle and raise important questions about the likely efficacy of mucosal immune responses against bacteria existing in different intestinal niches.

Serum antibody responses against the O157 lipopolysaccharide and Shiga toxin 1 have been detected in some but not all experimentally infected calves (Johnson *et al.*, 1996; Wray *et al.*, 2000) and sheep (Cornick *et al.*, 2000). Neutralizing antibody responses against Stx2 were not detected following *E. coli* O157:H7 infection of calves (Johnson *et al.*, 1996). Anti-LPS and Stx1 responses in calves could not be correlated with the elimination of *E. coli* O157:H7 and failed to reduce faecal shedding of the bacteria following re-inoculation with the same strain (Johnson *et al.*, 1996; Wray *et al.*, 2000). In contrast, Sanderson *et al.* (1999) reported that calves re-infected with *E. coli* O157:H7 shed the organism for a shorter duration following the second or third inoculation. Diarrhoeic calves experimentally infected with an O5 calf dysentery EHEC strain did not present with dysentery following a second challenge

(Chanter *et al.*, 1986). However, susceptibility of calves to this strain is age-related (Chanter *et al.*, 1986), therefore the lack of symptoms following re-inoculation may not necessarily be the result of acquired immunity. Further studies are required to measure the induction and efficacy of humoral and cell-mediated immune responses to EHEC following natural and experimental infection of ruminants.

The induction of intestinal secretory and inflammatory responses to different EHEC serotypes and defined bacterial mutants has been quantified in bovine ligated intestinal loop models (Stevens *et al.*, 2002a; Sandhu & Gyles, 2002). Whilst O157 strains were not strongly enteropathogenic, fluid accumulation and a pronounced infiltration of neutrophils was seen in response to EHEC serotypes O103 (Stevens *et al.*, 2002a) and O5, O26 and O111 (Sandhu & Gyles, 2002). This provides a valuable model to dissect the role of bacterial factors in EHEC-induced enteritis and intestinal colonization.

Factors mediating colonization of the bovine intestine by EHEC

EHEC are closely related to enteropathogenic *E. coli* (EPEC), which are a leading cause of infantile diarrhoea in developing countries, and share many of the EPEC genes implicated in virulence (Frankel *et al.*, 1998; Roe & Gally, 2000). EHEC induce a characteristic histopathology on intestinal epithelia known as the 'attaching and effacing' (AE) lesion (Nataro & Kaper, 1998). The bacteria adhere intimately to the apical surface of enterocytes on actin-rich pedestals and microvilli in the brush border are destroyed (Fig. 1). The genes that determine the AE phenotype have been mapped to a

chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE). The LEE encodes a type III protein secretion system that delivers a set of bacterial proteins into the host cell cytoplasm or apical membrane (Frankel *et al.*, 1998). In EPEC and EHEC, several proteins are exported by the type III secretion system, some of which subvert pathways that regulate the host cell cytoskeleton and bring about pedestal formation (Frankel *et al.*, 1998). One of the translocated proteins (Tir) becomes inserted in the apical membrane of enterocytes, where it acts as a receptor for a bacterial outer-membrane protein called intimin (Eae) (DeVinney *et al.*, 1999). Intimin can also bind to β 1-integrins (Frankel *et al.*, 1996) and to cell-surface localized nucleolin (Sinclair & O'Brien, 2002); however, the significance of this in intestinal colonization is not known.

The cell-binding activity of intimin has been localized to the carboxy-terminal 280 amino acids (Int280) of the polypeptide (Frankel *et al.*, 1994). Recently, several distinct Int280 subtypes, designated intimin α , β , γ , δ , ϵ and θ , have been identified which were specifically associated with distinct EPEC and EHEC serotypes (Adu-Bobie *et al.*, 1998). A further group of strains of different serotypes exists for which the intimin type was non-typable (NT). Different intimin subtypes confer a tropism for different intestinal sites (Phillips & Frankel, 2000). Intimin- γ (expressed by EHEC O157:H7) confers a specificity for follicle-associated epithelium overlying Peyer's patches in the human distal ileum (Phillips & Frankel, 2000). Expression of intimin- α (expressed by EPEC) in *E. coli* O157:H7 results in infection spreading to small intestinal mucosal surfaces (Fitzhenry *et al.*, 2002). Similar observations have been made in a

Fig. 1. (a) Confocal laser scanning micrograph showing extensive adherence of an O111:H⁻ EHEC strain to the epithelium of the bovine spiral colon. The tissue was analysed 3 days after oral inoculation of an 11-day-old conventional Friesian bull calf with 10^{10} c.f.u. of EHEC strain E45035N (Stevens *et al.*, 2002b). Bacteria are stained red with a fluorophore-conjugated antibody against the LPS and F-actin in the mucosa is stained green with fluorophore-conjugated phalloidin (bar, 10 μ m). (b) Transmission electron micrograph of the same tissue showing attaching and effacing (AE) lesions induced by the bacteria on colonic enterocytes (bar, 1 μ m).

gnotobiotic piglet model (Tzipori *et al.*, 1995). It is presently unclear if intimin-mediated tissue tropism correlates with the ability of some EHEC serotypes to successfully colonize both human and ruminant hosts.

Intimin has been shown to play a key role in intestinal colonization in colostrum-deprived neonatal calves and piglets by *E. coli* O157:H7 and in the induction of colonic oedema and diarrhoea (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Tzipori *et al.*, 1995; Dean-Nystrom *et al.*, 1998b). Intimin also influences colonization of adult cattle and sheep (Cornick *et al.*, 2002). However, the *eae* gene is not present in all Shiga-toxin-producing *E. coli* strains isolated from diarrhoeic calves and healthy cattle (Wieler *et al.*, 1996; Sandhu *et al.*, 1996; Jenkins *et al.*, 2002), indicating that other factors influencing persistence of the bacteria in the bovine intestine may be important. The finding that *eae* null mutants still colonize some compartments of the intestinal tract of calves and sheep (Dean-Nystrom *et al.*, 1998b; Cornick *et al.*, 2002) supports the notion that accessory adhesins and/or growth in the luminal contents may influence carriage. A number of loci have been implicated in the attachment of EHEC to epithelial cells *in vitro*, including LEE-encoded EspA filaments (Ebel *et al.*, 1998), Iha (Tarr *et al.*, 2000) and several other genes identified by transposon mutagenesis of *E. coli* O157:H7 (Tatsuno *et al.*, 2000). To our knowledge, the role of these genes in colonization of the ruminant intestine has not yet been tested.

LEE-negative Shiga-toxin-producing *E. coli* strains have also been implicated in outbreaks of human disease and HUS (Paton *et al.*, 1999). Recently, two factors encoded on the large plasmid of an O113:H21 LEE-negative isolate implicated in an outbreak of HUS were found to mediate attachment of the bacteria to cultured epithelial cells. One is an autoagglutinating adhesin (Saa) (Paton *et al.*, 2001) and the other is a novel type IV pilus (Srimanote *et al.*, 2002). The distribution of the *saa* and *pil* loci among LEE-negative EHEC strains isolated from ruminants and their role in intestinal colonization remains to be studied.

Recently, we have identified a gene (*efa1*) that mediates colonization of the bovine intestine by non-O157 EHEC (Stevens *et al.*, 2002b). Efa1 (EHEC factor for adherence) was recently implicated in adherence of a clinical O111:H⁻ EHEC strain to cultured Chinese Hamster Ovary cells (Nicholls *et al.*, 2000). Mutation of the *efa1* gene of EHEC serotypes O5 and O111 significantly reduced faecal shedding and bacterial adherence to the colonic epithelium in experimentally infected calves (Stevens *et al.*, 2002b). EHEC strains harbouring the *efa1* mutation were found to express and secrete slightly less of the LEE-encoded effector proteins (Stevens *et al.*, 2002b).

The *efa1* gene is present in all non-O157 strains so far tested (Nicholls *et al.*, 2000; Klapproth *et al.*, 2000), and is identical in size and 99.9% identical in nucleotide sequence to a gene in EPEC (*lifA*) encoding a lymphocyte inhibitory factor (lymphostatin). Lymphostatin confers

the ability to inhibit the proliferation of human peripheral blood lymphocytes and the mitogen-stimulated synthesis of interleukin-2, -4, -5, and interferon- γ (Klapproth *et al.*, 2000). The predicted product of *lifA* also inhibits the proliferation of human and murine gastrointestinal lymphocytes, indicating that it may modulate mucosal immunity in the gut (Malstrom & James, 1995; Klapproth *et al.*, 1996). EHEC strains can inhibit the proliferation of bovine peripheral blood monocytes, however it is not yet clear if this activity is attributed to the *efa1* gene (Stevens *et al.*, 2002b). Thus Efa1 may influence colonization of the bovine intestine in several ways. It may act as an adhesin per se, it may promote EHEC survival in the bovine gut by modulating mucosal immunity, or it may act indirectly by influencing the expression and secretion of LEE-encoded proteins or other membrane-associated proteins that influence colonization.

Whilst *E. coli* O157:H7 lacks the full-length *efa1* gene, analysis of the genome sequence has revealed that a truncated version of the *efa1* gene encoding the N-terminal 433 amino acids of the protein exists in the chromosome (Stevens *et al.*, 2002b). A transposon insertion upstream of the truncated *efa1* gene was recently shown to reduce bacterial adherence to human colon carcinoma cells (Tatsuno *et al.*, 2000). *E. coli* O157:H7 strains also encode a homologue of *efa1* (*toxB/l7095*) on the pO157 virulence plasmid (Nicholls *et al.*, 2000). *E. coli* O157:H7 strains containing derivatives of pO157 that lack *toxB* exhibit reduced adherence to cultured epithelial cells, possibly owing to a reduction in the production and secretion of LEE-encoded type III secreted proteins (Tatsuno *et al.*, 2001). The *toxB* gene has also been suggested to be a functional homologue of lymphostatin, since an *E. coli* O157:H7 strain cured of pO157 lacked the ability to inhibit IL-2 and IL-4 synthesis in mitogen-activated human PBMCs (Klapproth *et al.*, 2000). Thus the *E. coli* O157:H7 truncated *efa1* and *toxB* genes may influence intestinal colonization in ruminants in a similar manner to *efa1* in non-O157 EHEC.

The role of Shiga toxins in EHEC intestinal colonization and enteropathogenesis in ruminants is unclear. Nearly all *E. coli* O157 strains isolated from animals at slaughter in Great Britain contain the *stx2* gene (97.8% of cattle isolates and 100% of sheep isolates), and 19.9 and 1.4%, respectively, contained both *stx1* and *stx2* genes (Paiba *et al.*, 2002). This suggests a strong correlation between possession of *stx* genes and colonization of the ruminant host. Receptors for Stx have been detected on bovine intestinal crypt cells (Hoey *et al.*, 2002), and it has been reported that purified Stx1 can inhibit the activation and proliferation of bovine lymphocytes (Menge *et al.*, 1999; Ferens & Hovde, 2000). Thus Stx may facilitate intestinal colonization by modulating the mucosal immune response. Dean-Nystrom *et al.* (1998a) reported that an Stx-producing *E. coli* O157:H7 strain colonized weaned calves better than a non-producing strain; however, the strains used were not isogenic and differences in colonization may have been due to traits other

than Stx production. Stx1 does not appear to be involved in the induction of intestinal secretory and inflammatory responses to EHEC serotype O103:H2 in bovine ligated ileal loops (Stevens *et al.*, 2002a).

The role of flagella, fimbriae and other surface appendages in colonization of the ruminant intestine by EHEC remains to be tested. Flagella and/or motility are important for colonization, invasion and persistence in several enteric pathogens (La Ragione *et al.*, 2000; Allen-Vercoe *et al.*, 1999). The EPEC H6 flagellin was recently shown to mediate adherence to epithelial cells (Girón *et al.*, 2002); however, H7 flagella expressed by many *E. coli* O157 isolates lacked this activity. *E. coli* O157:H7 encodes 14 different fimbrial operons, however it is presently unclear which if any of these contribute to adherence. Indeed, *E. coli* O157 contains intact genes for the expression of type I fimbriae, however these are not expressed owing to a deletion in the *fimA* promoter (Roe *et al.*, 2001).

Insights from comparative and functional genomics in *E. coli* O157:H7

The complete genome sequences of two *E. coli* O157:H7 strains have been determined (Perna *et al.*, 2001; Hayashi *et al.*, 2001). Comparison of the genome sequence of strain EDL933 with that of the K-12 laboratory strain of *E. coli* has revealed that *E. coli* O157:H7 contains an extra 1.34 Mb of sequence encoding 1387 O157-specific genes organized mostly in 177 so-called O-islands (Perna *et al.*, 2001). Approximately 60% of O157-specific predicted genes have no known function. However, it has been possible to identify 14 different genes encoding known or putative non-fimbrial adhesins, a second putative type III protein secretion system similar to the *Salmonella* Inv/Spa apparatus, a large number of bacteriophage-related sequences and several putative toxins (Perna *et al.*, 2001; Hayashi *et al.*, 2001). This has provided researchers with valuable clues about how *E. coli* colonizes animal hosts and produces disease. Targeted disruption of candidate virulence genes and the testing of defined mutants in relevant experimental animal models is required to determine the role of these genes *in vivo*.

Given the size of the *E. coli* O157:H7 genome (approx. 5.5 Mb) and the large number of O157-specific genes, we have adopted a random mutagenesis strategy to identify EHEC genes required for colonization and survival in cattle using signature-tagged transposon mutagenesis (STM). STM makes it possible to track the fate of individually tagged bacterial transposon mutants in a complex pool during their interaction with the animal host (Hensel *et al.*, 1995). The composition of a pool of random mutants in the inoculum can be compared with the composition of a pool of mutants recovered from the faeces at time-points after oral inoculation of calves. This involves the use of PCR and DNA hybridization to detect oligonucleotide sequence tags unique to each mutant in the pool (Hensel *et al.*, 1995). In this way it is possible to identify EHEC transposon mutants deficient

in intestinal colonization and/or survival in the bovine host. Factors required for colonization can then be identified by cloning and sequencing of the disrupted genes. Recently we have shown that mini-Tn5Km2 insertions in genes encoding structural components of the LEE-encoded type III protein secretion system (*escC*, *escV* and *escN*) result in reduced faecal excretion of *E. coli* O157:H7 (P. van Diemen & F. Dziva, unpublished observations). This finding indicates that LEE-mediated adherence of *E. coli* O157:H7 to intestinal epithelia and AE-lesion formation is important for colonization and suggests that the bacteria are not merely transient members of the luminal microflora requiring continuous re-inoculation. The site(s) of specific adherence of *E. coli* O157:H7 to the intestinal epithelium in calves remain to be determined. LEE-encoded genes are also crucial for colonization of the rabbit intestine by rabbit EPEC (Abe *et al.*, 1998; Marchés *et al.*, 2000). Given that EHEC O157 and non-O157 serotypes potentially use different strategies to colonize the ruminant host we are also employing STM in an O26 calf dysentery isolate with recovery of bacterial mutants direct from the colonic mucosa. This may shed light on why some EHEC serotypes extensively interact with the intestinal epithelium whereas *E. coli* O157 apparently exists largely in the intestinal lumen. The information derived from screening tagged EHEC O157 and O26 transposon mutants in cattle will greatly facilitate the development of strategies to reduce the prevalence of EHEC in farm animal hosts. *E. coli* O157:H7 has been reported to undergo rapid genetic diversification following oral inoculation of calves with a single clonal isolate (Akiba *et al.*, 2000). It is presently unclear what effect this will have on the long-term efficacy of strategies to control EHEC.

Control by vaccination

As subclinical and clinical EHEC infections in ruminants are restricted to the intestinal epithelium and lumen it is likely that a vaccine must induce high titre specific secretory IgA responses at the mucosal surface and/or colostral IgG responses for passive protection of the neonate. Several studies to date suggest that intimin is a good candidate antigen for inclusion in an EHEC vaccine. In a murine model of EHEC infection using the related enteropathogen *Citrobacter rodentium*, purified Int280 could induce protection against *C. rodentium* infection following subcutaneous and mucosal vaccination (Ghaem-Maghani *et al.*, 2001). Recently Dean-Nystrom *et al.* (2002) reported that vaccination of pregnant pigs with intimin from *E. coli* O157:H7 gives rise to high intimin-specific antibody titres in the serum and colostrum. Neonatal piglets allowed to suckle vaccinated dams exhibited increased resistance to colonization and intestinal damage following experimental inoculation with *E. coli* O157:H7 compared to piglets allowed to suckle mock-vaccinated dams (Dean-Nystrom *et al.*, 2002). Recent observations by Yokomizo *et al.* (2002) and Ghaem-Maghani *et al.* (2001) suggest that it may be possible to improve the immunogenicity

of intimin subunit vaccines using bacterial toxins as adjuvants. Intranasal administration of a truncated version of *E. coli* O157:H7 intimin to calves treated with an adjuvant comprising a detoxified derivative of *E. coli* heat labile enterotoxin II induced elevated IgA-intimin specific antibody in nasal secretions and saliva as well as IgG1-specific antibody against intimin in the sera and colostrum of cows (Yokomizo *et al.*, 2002). To our knowledge, the protective efficacy of these responses against oral inoculation of calves with different EHEC serotypes has not yet been tested.

Immune responses against Int280 peptides are subtype-specific and do not protect against challenge with *C. rodentium* strains expressing heterologous intimin subtypes in the murine model (Ghaem-Maghami *et al.*, 2001). Immunization of mice with a conserved domain of intimin (Int388–667) was not effective. Thus intimin-based vaccines may have to incorporate several different Int280 subtypes in order to protect against the predominant EHEC serotypes in cattle (β , γ and ϵ). It is possible that vaccines based on the Efa1 colonization factor may avoid complications of subtype-specificity. The Efa1 protein is expressed at the bacterial cell surface (Nicholls *et al.*, 2000; M. Stevens, unpublished observations) and is present in all non-O157 EHEC strains tested to date (Nicholls *et al.*, 2000; Klapproth *et al.*, 2000). *E. coli* O157:H7 strains encode a truncated version of the *efa1* gene encoding the N-terminal domain of the protein. Thus subunit vaccines based on the N-terminus of Efa1 have the potential to elicit broadly reactive immunity against all EHEC serotypes.

Conjugate and toxoid vaccines based on the O157 lipopolysaccharide elicit specific antibody responses in mice and human volunteers (Konadu *et al.*, 1998, 1999; Conlan *et al.*, 1999a, 2000). However, oral and parenteral immunization of mice with an O157-glycoconjugate vaccine failed to protect against intestinal colonization with *E. coli* O157:H7 (Conlan *et al.*, 1999a, 2000). Calves make O-antigen-specific antibodies in response to *E. coli* O157 infection (Johnson *et al.*, 1996; Wray *et al.*, 2000); however, the induction of the antibody response could not be correlated with clearance of the bacteria.

Butterton *et al.* (1997) investigated the feasibility of using a live attenuated *Vibrio cholerae* El Tor vaccine strain (Peru2) expressing the *E. coli* O157:H7 intimin gene for protection against EHEC in a rabbit model. A plasmid construct for the expression of *eae* in Peru2 proved to be unstable and failed to elicit intimin-specific antibody responses following oro-gastric inoculation of rabbits (Butterton *et al.*, 1997). However, a Peru2 derivative with a chromosomal insertion of the *eae* gene induced a marked anti-Eae response in one of two rabbits. With development it should be possible to improve the expression of heterologous antigens in an appropriate bacterial carrier for cattle.

Previous studies have shown that oral vaccination of calves with a *Salmonella typhimurium* aro vaccine strain expressing fragment C of tetanus toxoid (TetC) induces

potent mucosal IgA and serum IgG responses against TetC (Villareal-Ramos *et al.*, 2000). Thus delivery of EHEC antigens by live attenuated *Salmonella* strains is a viable option to induce relevant EHEC-specific mucosal immunity. *Salmonella* strains expressing lipopolysaccharide O-antigens of the same serogroup as prevalent EHEC isolates (O157 and O111) have been developed and offer some protection in murine models (Conlan *et al.*, 1999b; Wang *et al.*, 1999). *Salmonella* carrier vaccines have the added advantage of inducing solid protection against virulent *Salmonella* strains in calves (Jones *et al.*, 1991). Live attenuated vaccines offer the advantage of *in vivo* amplification of the antigen, prolonged exposure of the mucosal immune system to the antigen and contact immunization via excreted organisms. However, such vaccines may be inhibited by competing organisms in the intestinal microflora and the presence of antibiotics or antibodies in the feed or milk. Further, live bacterial vaccines are unlikely to be used in food-producing animals until fears over safety and the potential for zoonotic transmission to humans are addressed. Kaper *et al.* (1998) suggested that 'edible vaccines' based on transgenic plants engineered to express intimin may offer another mode of delivery. Transgenic plant vaccines are capable of inducing substantial mucosal immune responses in murine models (Hag *et al.*, 1995).

Passive protection

Antibodies directed against the carboxy-terminal domain of intimin block adherence of *E. coli* O157:H7 to cultured epithelial cells (Gansheroff *et al.*, 1999) and mediate specific inhibition of colonization following immunization of mice with purified intimin (Ghaem-Maghami *et al.*, 2001). Use of colostrum from intimin-vaccinated pigs is effective in reducing EHEC colonization and pathogenesis in piglets (Dean-Nystrom *et al.*, 2002) and may reduce the prevalence of EHEC in younger animals. It is conceivable that recombinant antibodies directed against intimin or other EHEC colonization factors could be administered passively to food-producing farm animals or delivered in a plant vector to reduce the carriage of EHEC in the intestine prior to slaughter.

Probiotics and other inhibitors

Inhibition of EHEC proliferation in the ruminant gut may be mediated through competition for nutrients with endogenous or exogenous bacteria and/or toxic molecules. Inoculation of calves with a mixture of non-pathogenic *E. coli* strains and *Proteus mirabilis* has been reported to reduce rumen proliferation and faecal shedding of *E. coli* O157:H7 (Zhao *et al.*, 1998). Probiotic mixtures based on *Lactobacillus* and *Streptococcus* spp. have also shown considerable promise in reducing intestinal colonization and faecal shedding of *E. coli* O157:H7 in experimentally infected calves (Ohya *et al.*, 2000), sheep (Lema *et al.*, 2001) and infant rabbits (Ogawa *et al.*, 2001). Clearance of EHEC correlates with a marked increase in the presence of volatile fatty acids

following inoculation with probiotic bacteria and occurred in young animals with a relatively undeveloped gut microflora. In adult ruminants lactic-acid-producing bacteria are a very minor constituent of the intestinal microflora and so may be less effective or require frequent re-inoculation. Nevertheless, administration of probiotic bacteria to reduce the carriage of EHEC prior to slaughter may significantly reduce the introduction of EHEC into the human food chain. *Pseudomonas aeruginosa* strains inhibitory to *E. coli* O157:H7 have been isolated from the rumens of sheep (Duncan *et al.*, 1999); however, to our knowledge the ability of these bacteria to inhibit EHEC infection in ruminants has not been tested. Lytic bacteriophages that specifically target EHEC expressing the O157 antigen and colicins active on EHEC have also been isolated and may be useful for the control of *E. coli* O157:H7 in ruminants prior to slaughter (Murinda *et al.*, 1996; Kudva *et al.*, 1999). It is unlikely that such agents could be effective long term owing to the development of genetic resistance. The possible use of non-therapeutic compounds to create an intestinal environment unfavourable to EHEC is reviewed elsewhere (Duncan *et al.*, 2000).

Effect of diet and fasting

Several studies indicate that it may be possible to reduce the carriage of EHEC by calves and sheep by manipulating the diet. Diet and the feeding regime may influence growth and survival of EHEC in the ruminant intestine by creating fermentation conditions that give rise to elevated volatile fatty acid concentrations, adverse pH and/or changes in the composition of the resident microflora (Duncan *et al.*, 2000; Russell *et al.*, 2000). Further, different diets may influence the magnitude and duration of shedding of EHEC by altering the rate of epithelial cell proliferation in the lower gastrointestinal tract (Magnuson *et al.*, 2000).

Reports on the effect of diet and diet shifts on shedding of *E. coli* O157:H7 in ruminants are often conflicting. Grain feeding has been proposed as a risk factor for increased shedding of EHEC in cattle (Diez-Gonzalez *et al.*, 1998; Dargatz *et al.*, 1997). However, Hancock *et al.* (1994) and Magnuson *et al.* (2000) found no difference in the amount or duration of *E. coli* O157:H7 shedding between animals fed grain and forage diets. Indeed, grain-fed sheep shed *E. coli* O157:H7 for a significantly shorter duration under some conditions than sheep fed hay (Kudva *et al.*, 1997). Experimentally inoculated calves fed on a primarily grain-based diet develop a lower rumen pH and shed elevated numbers of acid-tolerant *E. coli* O157:H7 than calves fed on a high-roughage (hay-based) diet (Diez-Gonzalez *et al.*, 1998; Tkalcic *et al.*, 2000). Acid-tolerant EHEC may be expected to colonize other animals, and possibly humans, more efficiently since they may survive better in the acidic compartments of the intestine. Thus grain diets may actually facilitate transmission of the bacteria in the farm environment. A brief period of feeding hay to grain-fed calves has been reported to decrease the number of acid-resistant *E. coli* O157:H7 shed sub-

stantially (Diez-Gonzalez *et al.*, 1998) and may help to reduce entry of acid-resistant EHEC into the food chain. However, Hovde *et al.* (1999) found that hay-fed animals shed *E. coli* O157:H7 for longer than grain-fed animals, and that irrespective of diet, equal numbers of acid-resistant EHEC were shed. Further studies are required to determine the influence of different diets on the shedding and infectivity of EHEC in ruminants.

Other foodstuffs including cottonseed, clover or lucerne hay and soymeal have been reported to reduce the shedding of *E. coli* in cattle (Brownlie & Grau, 1967; Dargatz *et al.*, 1997; Garber *et al.*, 1995), while barley (Dargatz *et al.*, 1997), corn silage (Herriott *et al.*, 1998) and beetpulp (Schouten *et al.*, 2001) appear to increase carriage rates. The relative abundance in plants of secondary metabolites inhibitory to EHEC may play a role in this (Duncan *et al.*, 2000).

Fasting is common before and during transportation of animals to abattoirs and is intended to reduce soiling of the hide. This practice may inadvertently increase the proliferation of EHEC in the intestine by causing the rumen pH to rise and the concentration of volatile fatty acids to drop (Duncan *et al.*, 2000). Cray *et al.* (1998) reported that withholding food for 48 h prior to inoculation rendered calves more susceptible to *E. coli* O157:H7 infection and that significantly more bacteria were shed compared with animals fed without interruption. Similar observations have been made in rams deprived of feed and water (Kudva *et al.*, 1995). Withdrawal of food from calves carrying EHEC has been shown to increase faecal shedding in some previously low-shedding calves (Brown *et al.*, 1997), and was variable in calves shedding larger populations (Brown *et al.*, 1997). However, Cray *et al.* (1998) found no effect on shedding levels of withdrawing feed after *E. coli* O157:H7 infection of calves. An important consideration when comparing the results of such studies may be the accuracy of quantification of EHEC excretion rates and the possible influence of water content on the count of bacteria per unit weight of sample.

Impact of farm management practices

Considerable effort has been expended to identify herd management practices and environmental factors that inhibit or facilitate infection of animals with *E. coli* O157:H7 (reviewed by Hancock *et al.*, 1998). EHEC transmit readily between ruminants in the farm environment (Besser *et al.*, 2001), and wild animals may represent important vectors. Environmental factors that may influence *E. coli* O157:H7 shedding in cattle include livestock feed (see above) and waste-handling practices (Kudva *et al.*, 1998), presence of non-farm animals in the location of the herd (Schouten *et al.*, 2001) as well as insects and microbial interactions in soil and water (Rasmussen & Casey, 2001). Provision of potable water, clean feed, adequate bedding and clean accommodation should help to reduce within-herd transmission of EHEC. Measures to control EHEC at slaughter are described elsewhere (Gannon, 1999). Elimination of

EHEC by testing and removal of infected animals would appear to be impractical since the majority of herds are infected at some time.

Perspective

Although the incidence of human EHEC infection is relatively low, the severity of the symptoms and frequency of renal and neurological sequelae give cause for concern. The disease has a high public profile and there is a pressing demand for the development of treatment and/or prevention strategies. Considerable progress has been made in understanding the ecology of EHEC in farm animal hosts and the modes of transmission to humans. It remains unclear if all EHEC present in animal reservoirs present a risk to human health and further studies are required on the genetic stability of EHEC during their interaction with ruminants. Strategies to reduce the prevalence of EHEC in ruminants offer the potential to lower the incidence of human infection. Probiotics, bacteriophages and feeding regimes that create an intestinal environment inhibitory to EHEC may prove to be useful prior to slaughter to reduce the entry of EHEC into the food chain. Given the extremely low infectious dose for humans, it is open to question whether such strategies can reduce the shedding of EHEC by the amount needed to significantly reduce the risk to human health. Further research is required on the mechanisms underlying EHEC-intestinal colonization and enteropathogenesis in cattle and sheep and the host responses to infection. Insights gained from the genome sequence of *E. coli* O157:H7 and from screening defined and signature-tagged transposon EHEC mutants in experimental animal models will add greatly to our understanding of these processes. This information will be crucial for the design of vaccines or other intervention measures.

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References

- Abe, A., Heczko, U., Hegele, H. G. & Finlay, B. B. (1998). Two enteropathogenic *Escherichia coli* type III secreted proteins, EspA and EspB, are virulence factors. *J Exp Med* **188**, 1907–1916.
- Adu-Bobie, J., Frankel, G., Bain, C., Goncalves, A. G., Trabulsi, L. R., Douce, G., Knutton, S. & Dougan, G. (1998). Detection of intimin α , β , γ , and δ , four derivatives expressed by attaching and effacing microbial pathogens. *J Clin Microbiol* **36**, 662–668.
- Akiba, M., Sameshima, T. & Nakazawa, M. (2000). Clonal turnover of enterohemorrhagic *Escherichia coli* O157:H7 in experimentally infected cattle. *FEMS Microbiol Lett* **184**, 79–83.
- Allen-Vercoe, E., Sayers, A. R. & Woodward, M. J. (1999). Virulence of *Salmonella enterica* serotype Enteritidis aflagellate and afimbriate mutants in a day-old chick model. *Epidemiol Infect* **122**, 395–402.
- Anon (1998). Vero cytotoxin producing *Escherichia coli* in England and Wales. *Commun Dis Rep Wkly* **8**, 169.

- Anon (2000). Surveillance of vero cytotoxin producing *Escherichia coli* O157 in England and Wales. *Commun Dis Rep Wkly* **10**, 171.
- Armstrong, G. L., Hollingsworth, J. & Morris, J. G., Jr (1996). Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol Rev* **18**, 29–51.
- Besser, T. E., Richards, B. L., Rice, D. H. & Hancock, D. D. (2001). *Escherichia coli* O157:H7 infection of calves: infectious dose and direct contact transmission. *Epidemiol Infect* **127**, 555–560.
- Bettelheim, K. A. (2000). Role of non-O157 VTEC. *Symp Ser Soc Appl Microbiol* **29**, 38S–50S.
- Brown, C. A., Harmon, B. G., Zhao, T. & Doyle, M. P. (1997). Experimental *Escherichia coli* O157:H7 carriage in calves. *Appl Environ Microbiol* **63**, 27–32.
- Brownlie, L. E. & Grau, F. H. (1967). Effect of food intake on the growth and survival of *Salmonellas* and *Escherichia coli* in the bovine rumen. *J Gen Microbiol* **46**, 125–134.
- Butterton, J. R., Ryan, E. T., Acheson, D. W. & Calderwood, S. B. (1997). Coexpression of the B subunit of Shiga toxin 1 and EaeA from enterohemorrhagic *Escherichia coli* in *Vibrio cholerae* vaccine strains. *Infect Immun* **65**, 2127–2135.
- Chanter, N., Hall, G. A., Bland, A. P., Hayle, A. J. & Parsons, K. R. (1986). Dysentery in calves caused by an atypical strain of *Escherichia coli* (S102-9). *Vet Microbiol* **12**, 241–253.
- Cody, S. H., Glynn, M. K., Farrar, J. A. & 10 other authors (1999). An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann Intern Med* **130**, 202–209.
- Conlan, J. W., Cox, A. D., KuoLee, R., Webb, A. & Perry, M. B. (1999a). Parenteral immunization with a glycoconjugate vaccine containing the O157 antigen of *Escherichia coli* O157:H7 elicits a systemic humoral immune response in mice, but fails to prevent colonization by the pathogen. *Can J Microbiol* **45**, 279–286.
- Conlan, J. W., KuoLee, R., Webb, A. & Perry, M. B. (1999b). *Salmonella landau* as a live vaccine against *Escherichia coli* O157:H7 investigated in a mouse model of intestinal colonization. *Can J Microbiol* **45**, 723–731.
- Conlan, J. W., KuoLee, R., Webb, A., Cox, A. D. & Perry, M. B. (2000). Oral immunization of mice with a glycoconjugate vaccine containing the O157 antigen of *Escherichia coli* O157:H7 admixed with cholera toxin fails to elicit protection against subsequent colonization by the pathogen. *Can J Microbiol* **46**, 283–290.
- Cornick, N. A., Booher, S. L., Casey, T. A. & Moon, H. W. (2000). Persistent colonization of sheep by *Escherichia coli* O157:H7 and other *E. coli* pathotypes. *Appl Environ Microbiol* **66**, 4926–4934.
- Cornick, N. A., Booher, S. L. & Moon, H. W. (2002). Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. *Infect Immun* **70**, 2704–2707.
- Cray, W. C., Jr & Moon, H. W. (1995). Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl Environ Microbiol* **61**, 1586–1590.
- Cray, W. C., Jr, Casey, T. A., Bosworth, B. T. & Rasmussen, M. A. (1998). Effect of dietary stress on fecal shedding of *Escherichia coli* O157:H7 in calves. *Appl Environ Microbiol* **64**, 1975–1979.
- Dargatz, D. A., Wells, S. J., Thomas, L. A., Hancock, D. D. & Garber, L. P. (1997). Factors associated with the presence of *Escherichia coli* O157 in faeces of feedlot cattle. *J Food Protein* **5**, 466–470.
- Dean-Nystrom, E. A., Bosworth, B. T., Cray, W. C., Jr & Moon, H. W. (1997). Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infect Immun* **65**, 1842–1848.

- Dean-Nystrom, E. A., Bosworth, B. T., Moon, H. W. & O'Brien, A. D. (1998a). Bovine infection with Shiga toxin-producing *Escherichia coli*. In *Escherichia coli* O157:H7 and Other Shiga Toxin-producing *Escherichia coli*, pp. 261–267. Edited by J. B. Kaper & A. D. O'Brien. Washington, DC: American Society for Microbiology.
- Dean-Nystrom, E. A., Bosworth, B. T., Moon, H. W. & O'Brien, A. D. (1998b). *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. *Infect Immun* 66, 4560–4563.
- Dean-Nystrom, E. A., Bosworth, B. T. & Moon, H. W. (1999). Pathogenesis of *Escherichia coli* O157:H7 in weaned calves. *Adv Exp Med Biol* 473, 173–177.
- Dean-Nystrom, E. A., Gansheroff, L. J., Mills, M., Moon, H. W. & O'Brien, A. D. (2002). Vaccination of pregnant dams with intimin(O157) protects suckling piglets from *Escherichia coli* O157:H7 infection. *Infect Immun* 70, 2414–2418.
- DeVinney, R., Stein, M., Reinscheid, D., Abe, A., Ruschkowski, S. & Finlay, B. B. (1999). Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. *Infect Immun* 67, 2389–2398.
- Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G. & Russell, J. B. (1998). Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 281, 1666–1668.
- Donnenberg, M. S., Tzipori, S. S., McKee, M. L., O'Brien, A. D., Alroy, J. & Kaper, J. B. (1993). Role of the *eae* gene of enterohaemorrhagic *Escherichia coli* in intimate attachment *in vitro* and in a porcine model. *J Clin Invest* 92, 1418–1424.
- Duncan, S. H., Doherty, C. J., Govan, J. R., Neogrady, S., Galfi, P. & Stewart, C. S. (1999). Characteristics of sheep-rumen isolates of *Pseudomonas aeruginosa* inhibitory to the growth of *Escherichia coli* O157. *FEMS Microbiol Lett* 180, 305–310.
- Duncan, S. H., Booth, I. R., Flint, H. J. & Stewart, C. S. (2000). The potential for the control of *Escherichia coli* O157 in farm animals. *Symp Ser Soc Appl Microbiol* 29, 157S–165S.
- Ebel, F., Podzadel, T., Rohde, M., Kresse, A. U., Krämer, S., Deibel, C., Guzmán, C. A. & Chakraborty, T. (1998). Initial binding of Shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. *Mol Microbiol* 30, 147–161.
- Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, G. A., Koohmaraie, M. & Laegreid, W. W. (2000). Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci USA* 97, 2999–3003.
- Ferens, W. A. & Hovde, C. J. (2000). Antiviral activity of Shiga toxin 1: suppression of bovine leukemia virus-related spontaneous lymphocyte proliferation. *Infect Immun* 68, 4462–4469.
- Fitzhenry, R. J., Pickard, D. J., Hartland, E. L., Reece, S., Dougan, G., Phillips, A. D. & Frankel, G. (2002). Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. *Gut* 50, 180–185.
- Frankel, G., Candy, D. C., Everest, P. & Dougan, G. (1994). Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. *Infect Immun* 62, 1835–1842.
- Frankel, G., Lider, O., Hershkoviz, R., Mould, A. P., Kachalsky, S. G., Candy, D. C. A., Cahalon, L., Humphries, M. J. & Dougan, G. (1996). The cell-binding domain of intimin from enteropathogenic *Escherichia coli* binds to beta1 integrins. *J Biol Chem* 271, 20359–20364.
- Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* 30, 911–921.
- Gannon, V. P. J. (1999). Control of *Escherichia coli* at slaughter. In *Escherichia coli* O157 in Farm Animals, pp. 169–193. Edited by C. S. Stewart & H. J. Flint. Wallingford: CAB International.
- Gansheroff, L. J. & O'Brien, A. D. (2000). *Escherichia coli* O157:H7 in beef cattle presented for slaughter in the U.S.: higher prevalence rates than previously estimated. *Proc Natl Acad Sci USA* 97, 2959–2961.
- Gansheroff, L. J., Wachtel, M. R. & O'Brien, A. D. (1999). Decreased adherence of enterohemorrhagic *Escherichia coli* to HEP-2 cells in the presence of antibodies that recognize the C-terminal region of intimin. *Infect Immun* 67, 6409–6417.
- Garber, L. P., Wells, S. J., Hancock, D. D., Doyle, M. P., Tuttle, J., Shere, J. A. & Zhao, T. (1995). Risk factors for fecal shedding of *Escherichia coli* O157:H7 in dairy calves. *J Am Vet Med Assoc* 207, 46–49.
- Ghaem-Maghami, M., Simmons, C. P., Daniell, S., Pizza, M., Lewis, D., Frankel, G. & Dougan, G. (2001). Intimin-specific immune responses prevent bacterial colonization by the attaching-effacing pathogen *Citrobacter rodentium*. *Infect Immun* 69, 5597–5605.
- Girón, J. A., Torres, A. G., Freer, E. & Kaper, J. B. (2002). The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol* 44, 361–379.
- Grauke, L. J., Kudva, I. T., Yoon, J. W., Hunt, C. W., Williams, C. J. & Hovde, C. J. (2002). Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. *Appl Environ Microbiol* 68, 2269–2277.
- Griffin, P. M. (1995). *Escherichia coli* O157:H7 and other enterohaemorrhagic *Escherichia coli*. In *Infections of the Gastrointestinal Tract*, pp. 739–761. Edited by M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg & R. L. Guerrant. New York: Raven Press.
- Hall, G. A., Reynolds, D. J., Chanter, N., Morgan, J. H., Parsons, K. R., Debney, T. G., Bland, A. P. & Bridger, J. C. (1985). Dysentery caused by *Escherichia coli* (S102-9) in calves: natural and experimental disease. *Vet Pathol* 22, 156–163.
- Hancock, D. D., Besser, T. E., Kinsel, M. L., Tarr, P. I., Rice, D. H. & Paros, M. G. (1994). The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington State. *Epidemiol Infect* 113, 119–207.
- Hancock, D. D., Besser, T. E. & Rice, D. H. (1998). Ecology of *Escherichia coli* O157:H7 in cattle and impact of management practices. In *Escherichia coli* O157:H7 and other Shiga Toxin-producing *Escherichia coli*, pp. 85–91. Edited by J. B. Kaper & A. D. O'Brien. Washington, DC: American Society for Microbiology.
- Haq, T. A., Mason, H. S., Clements, J. D. & Arntzen, C. J. (1995). Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268, 714–716.
- Hayashi, T., Makino, K., Ohnishi, M. & 19 other authors (2001). Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res* 8, 11–22.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. *Science* 269, 400–403.
- Herriott, D. E., Hancock, D. D., Ebel, E. D., Carpenter, L. V., Rice, D. H. & Besser, T. E. (1998). Association of herd management

- factors with colonization of dairy cattle by Shiga toxin-positive *Escherichia coli* O157. *J Food Protein* **61**, 802–807.
- Hilborn, E. D., Mermin, J. H., Mshar, P. A. & 9 other authors (1999). A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med* **159**, 1758–1764.
- Hoey, D. E., Currie, C., Else, R. W., Nutikka, A., Lingwood, C. A., Gally, D. L. & Smith, D. G. (2002). Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. *J Med Microbiol* **51**, 143–149.
- Hovde, C. J., Austin, P. R., Cloud, K. A., William, C. J. & Hunt, C. W. (1999). Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Appl Environ Microbiol* **65**, 3233–3235.
- Jenkins, C., Chart, H., Cheasty, T. & 8 other authors (2002). Verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 from Scottish cattle. *Vet Rec* **15**, 58–60.
- Johnson, R. P., Cray, W. C., Jr & Johnson, S. T. (1996). Serum antibody responses of cattle following experimental infection with *Escherichia coli* O157:H7. *Infect Immun* **64**, 1879–1883.
- Jones, P. W., Dougan, G., Hayward, C., Mackenzie, N., Collins, P. & Chatfield, S. N. (1991). Oral vaccination of calves against experimental salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. *Vaccine* **9**, 29–34.
- Kaper, J. B., Gansheroff, L. J., Wachtel, M. R. & O'Brien, A. D. (1998). Intimin-mediated adherence of Shiga toxin-producing *Escherichia coli* and attaching-and-effacing pathogens. In *Escherichia coli* O157:H7 and Other Shiga Toxin-producing *Escherichia coli*, pp. 148–156. Edited by J. B. Kaper & A. D. O'Brien. Washington, DC: American Society for Microbiology.
- Klapproth, J. M., Donnenberg, M. S., Abraham, J. M. & James, S. P. (1996). Products of enteropathogenic *E. coli* inhibit lymphokine production by gastrointestinal lymphocytes. *Am J Physiol* **271**, G841–848.
- Klapproth, J. M., Scaletsky, I. C., McNamara, B. P., Lai, L. C., Malstrom, C., James, S. P. & Donnenberg, M. S. (2000). A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. *Infect Immun* **68**, 2148–2155.
- Kobayashi, H., Shimada, J., Nakazawa, M., Morozumi, T., Pohjanvirta, T., Pelkonen, S. & Yamamoto, K. (2001). Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. *Appl Environ Microbiol* **67**, 484–489.
- Konadu, E. Y., Parke, J. C., Jr, Tran, H. T., Bryla, D. A., Robbins, J. B. & Szu, S. C. (1998). Investigational vaccine for *Escherichia coli* O157: phase 1 study of O157 O-specific polysaccharide-*Pseudomonas aeruginosa* recombinant exoprotein A conjugates in adults. *J Infect Dis* **177**, 383–387.
- Konadu, E., Donohue-Rolfe, A., Calderwood, S. B., Pozsgay, V., Shiloach, J., Robbins, J. B. & Szu, S. C. (1999). Syntheses and immunologic properties of *Escherichia coli* O157 O-specific polysaccharide and Shiga toxin 1 B subunit conjugates in mice. *Infect Immun* **67**, 6191–6193.
- Kudva, I. T., Hatfield, P. G. & Hovde, C. J. (1995). Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model. *Appl Environ Microbiol* **61**, 1363–1370.
- Kudva, I. T., Hunt, C. W., Williams, C. J., Nance, U. M. & Hovde, C. J. (1997). Evaluation of dietary influences on *Escherichia coli* O157:H7 shedding by sheep. *Appl Environ Microbiol* **63**, 3878–3886.
- Kudva, I. T., Blanch, K. & Hovde, C. J. (1998). Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Appl Environ Microbiol* **64**, 3166–3174.
- Kudva, I. T., Jelacic, S., Tarr, P. I., Youderian, P. & Hovde, C. J. (1999). Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Appl Environ Microbiol* **65**, 3767–3773.
- La Ragione, R. M., Sayers, A. R. & Woodward, M. J. (2000). The role of fimbriae and flagella in the colonization, invasion and persistence of *Escherichia coli* O78:K80 in the day-old-chick model. *Epidemiol Infect* **124**, 351–363.
- Lema, M., Williams, L. & Rao, D. R. (2001). Reduction of fecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in lambs by feeding microbial feed supplement. *Small Ruminant Res* **39**, 31–39.
- Locking, M. E., O'Brien, S. J., Reilly, W. J., Wright, E. M., Campbell, D. M., Coia, J. E., Browning, L. M. & Ramsay, C. N. (2001). Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. *Epidemiol Infect* **127**, 215–220.
- Magnuson, B. A., Davis, M., Hubele, S., Austin, P. R., Kudva, I. T., Williams, C. J., Hunt, C. W. & Hovde, C. J. (2000). Ruminant gastrointestinal cell proliferation and clearance of *Escherichia coli* O157:H7. *Infect Immun* **68**, 3808–3814.
- Mainil, J. (1999). Shiga/verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals. *Vet Res* **30**, 235–257.
- Malstrom, C. & James, S. (1995). Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infect Immun* **66**, 3120–3127.
- Marchés, O., Nougayrède, J.-P., Boullier, S. & 8 other authors (2000). Role of Tir and intimin in the virulence of rabbit enteropathogenic *Escherichia coli* serotype O103:H2. *Infect Immun* **68**, 2171–2182.
- McKee, M. L., Melton-Celsa, A. R., Moxley, R. A., Francis, D. H. & O'Brien, A. D. (1995). Enterohaemorrhagic *Escherichia coli* O157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells. *Infect Immun* **63**, 3739–3744.
- McNally, A., Roe, A. J., Simpson, S., Thomson-Carter, F. M., Hoey, D. E., Currie, C., Chakraborty, T., Smith, D. G. & Gally, D. L. (2001). Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. *Infect Immun* **69**, 5107–5114.
- Menge, C., Wieler, L. H., Schlapp, T. & Baljer, G. (1999). Shiga toxin 1 from *Escherichia coli* blocks activation and proliferation of bovine lymphocyte subpopulations *in vitro*. *Infect Immun* **67**, 2209–2217.
- Murinda, S. E., Roberts, R. F. & Wilson, R. A. (1996). Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. *Appl Environ Microbiol* **62**, 3196–3202.
- Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142–201.
- Nicholls, L., Grant, T. H. & Robins-Browne, R. M. (2000). Identification of a novel genetic locus that is required for *in vitro* adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. *Mol Microbiol* **35**, 275–288.
- O'Brien, S. J., Adak, G. K. & Gilham, C. (2001). Contact with farming environment as a major risk factor for Shiga toxin (Verocytotoxin)-producing *Escherichia coli* O157 infection in humans. *Emerg Infect Dis* **7**, 1049–1051.
- Ogawa, M., Shimizu, K., Nomoto, K. & 7 other authors (2001). Protective effect of *Lactobacillus casei* strain Shirota on Shiga toxin-producing *Escherichia coli* O157:H7 infection in infant rabbits. *Infect Immun* **69**, 1101–1108.
- Ogden, I. D., Hepburn, N. F., MacRae, M., Strachan, N. J., Fenlon, D. R., Rusbridge, S. M. & Pennington, T. H. (2002). Long-term

- survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Lett Appl Microbiol* 34, 100–104.
- Ohya, T., Marubashi, T. & Ito, H. (2000). Significance of fecal volatile fatty acids in shedding of *Escherichia coli* O157 from calves: experimental infection and preliminary use of a probiotic product. *J Vet Med Sci* 62, 1151–1155.
- Olsen, S. J., Miller, G., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W. & Mead, P. (2002). A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. *Emerg Infect Dis* 8, 370–375.
- Paiba, G. A., Gibbens, J. C., Pascoe, S. J. S. & 12 other authors (2002). Faecal carriage of verotoxin-producing *Escherichia coli* O157 in cattle and sheep at slaughter in Great Britain. *Vet Rec* 150, 593–598.
- Paton, A. W., Woodrow, M. C., Doyle, R. M., Lanser, J. A. & Paton, J. C. (1999). Molecular characterization of a Shiga toxin-producing *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J Clin Microbiol* 37, 3357–3361.
- Paton, A. W., Srimanote, P., Woodrow, M. C. & Paton, J. C. (2001). Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxinogenic *Escherichia coli* strains that are virulent for humans. *Infect Immun* 69, 6999–7009.
- Paton, J. C. & Paton, A. W. (1998). Pathogenesis and diagnosis of Shiga-toxin producing *Escherichia coli* infections. *Clin Microbiol Rev* 11, 450–479.
- Pearson, G. R., Bazeley, K. J., Jones, J. R., Gunning, R. F., Green, M. J., Cookson, A. & Woodward, M. J. (1999). Attaching and effacing lesions in the large intestine of an eight-month-old heifer associated with *Escherichia coli* O26 infection in a group of animals with dysentery. *Vet Rec* 145, 370–373.
- Perna, N. T., Plunkett, G., Burland, V. & 25 other authors (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533.
- Phillips, A. D. & Frankel, G. (2000). Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. *J Infect Dis* 181, 1496–1500.
- Pritchard, G. C., Williamson, S., Carson, T., Bailey, J. R., Warne, L., Willshaw, G. & Cheasty, T. (2001). Wild rabbits – a novel vector for verocytotoxinogenic *Escherichia coli* O157. *Vet Rec* 149, 567.
- Rasmussen, M. A. & Casey, T. A. (2001). Environmental and food safety aspects of *Escherichia coli* O157:H7 infections in cattle. *Crit Rev Microbiol* 27, 57–73.
- Roe, A. J. & Gally, D. L. (2000). Enteropathogenic and enterohaemorrhagic *Escherichia coli* and diarrhoea. *Curr Opin Infect Dis* 13, 511–517.
- Roe, A. J., Currie, C., Smith, D. G. & Gally, D. L. (2001). Analysis of type 1 fimbriae expression in verotoxinogenic *Escherichia coli*: a comparison between serotypes O157 and O26. *Microbiology* 147, 145–152.
- Russell, J. B., Diez-Gonzalez, F. & Jarvis, G. N. (2000). Potential effect of cattle diets on the transmission of pathogenic *Escherichia coli* to humans. *Microbes Infect* 2, 45–53.
- Sanderson, M. W., Besser, T. E., Gay, J. M., Gay, C. C. & Hancock, D. D. (1999). Fecal *Escherichia coli* O157:H7 shedding patterns of orally inoculated calves. *Vet Microbiol* 69, 199–205.
- Sandhu, K. S. & Gyles, C. L. (2002). Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. *Can J Vet Res* 66, 65–72.
- Sandhu, K. S., Clarke, R. C., McFadden, K., Brouwer, A., Louie, M., Wilson, J., Loir, H. & Gyles, C. L. (1996). Prevalence of the *eaeA* gene in verotoxinogenic *Escherichia coli* strains from dairy cattle in Southwest Ontario. *Epidemiol Infect* 116, 1–7.
- Schouten, J. M., Bouwknegt, M., van de Giessen, A. W., Frankena, K. & Graat, E. A. M. (2001). Risk factor analysis of *Escherichia coli* O157 on Dutch dairy farms: preliminary results. In *Proceedings of the Society for Veterinary Epidemiology and Preventive Medicine*, pp. 202–210. Edited by F. D. Menzies & S. W. J. Reid. Noordwijkerhout, NL.
- Sinclair, J. F. & O'Brien, A. D. (2002). Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. *J Biol Chem* 277, 2876–2885.
- Slutsker, L., Ries, A. A., Greene, K. D., Wells, J. G., Hutwagner, L. & Griffin, P. M. (1997). *Escherichia coli* O157:H7 diarrhea in the United States: clinical and epidemiological features. *Ann Intern Med* 126, 505–513.
- Srimanote, P., Paton, A. W. & Paton, J. C. (2002). Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative Shiga-toxinogenic *Escherichia coli* strains that are virulent for humans. *Infect Immun* 70, 3094–3100.
- Stevens, M. P., Marchés, O., Campbell, J., Huter, V., Frankel, G., Phillips, A. D., Oswald, E. & Wallis, T. S. (2002a). Intimin, Tir and Shiga toxin 1 do not influence enteropathogenic responses to Shiga toxin-producing *Escherichia coli* in bovine ligated intestinal loops. *Infect Immun* 70, 945–952.
- Stevens, M. P., van Diemen, P. M., Frankel, G., Phillips, A. D. & Wallis, T. S. (2002b). Efa1 influences colonisation of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infect Immun* 70, 5158–5166.
- Tarr, P. I., Bilge, S. S., Vary, J. C., Jr, Jelacic, S., Habbab, R. L., Ward, T. R., Baylor, M. R. & Besser, T. E. (2000). Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect Immun* 68, 1400–1407.
- Tatsuno, I., Kimura, H., Okutani, A. & 10 other authors (2000). Isolation and characterization of mini-Tn5Km2 insertion mutants of enterohemorrhagic *Escherichia coli* O157:H7 deficient in adherence to Caco-2 cells. *Infect Immun* 68, 5943–5952.
- Tatsuno, I., Horie, M., Abe, H. & 7 other authors (2001). *toxB* gene on pO157 of enterohaemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infect Immun* 69, 6660–6669.
- Tkalcic, S., Brown, C. A., Harmon, B. G. & 7 other authors (2000). Effects of diet on rumen proliferation and fecal shedding of *Escherichia coli* O157:H7 in calves. *J Food Protein* 63, 1630–1636.
- Tzipori, S., Gunzer, F., Donnenberg, M. S., de Montigny, L., Kaper, J. B. & Donohue-Rolfe, A. (1995). The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohaemorrhagic *Escherichia coli* infection. *Infect Immun* 63, 3621–3627.
- Villarreal-Ramos, B., Manser, J. M., Collins, R. A., Dougan, G. & Howard, C. J. (2000). Cattle immune responses to tetanus toxoid elicited by recombinant *S. typhimurium* vaccines or tetanus toxoid in alum or Freund's adjuvant. *Vaccine* 18, 1515–1521.
- Wales, A. D., Clifton-Hadley, F. A., Cookson, A. L., Dibb-Fuller, M. P., La Ragione, R. M., Sprigings, K. A., Pearson, G. R. & Woodward, M. J. (2001a). Experimental infection of six-month-old sheep with *Escherichia coli* O157:H7. *Vet Rec* 148, 630–631.
- Wales, A. D., Pearson, G. R., Skuse, A. M., Roe, J. M., Hayes,

- C. M., Cookson, A. L. & Woodward, M. J. (2001b).** Attaching and effacing lesions caused by *Escherichia coli* O157:H7 in experimentally inoculated neonatal lambs. *J Med Microbiol* **50**, 752–758.
- Wang, L., Curd, H. & Reeves, P. R. (1999).** Immunization of mice with live oral vaccine based on a *Salmonella enterica* (sv Typhimurium) *aroA* strain expressing the *Escherichia coli* O111 O antigen. *Microb Pathog* **27**, 55–59.
- Wieler, L. H., Vieler, E., Erpestein, C., Schlapp, T., Steinrück, H., Bauerfeind, R., Byomi, A. & Baljer, G. (1996).** Shiga toxin-producing *Escherichia coli* strains from bovines: association of adhesion with carriage of *eae* and other genes. *Infect Immun* **34**, 2980–2984.
- Woodward, M. J., Gavier-Widen, D., McLaren, I. M., Wray, C., Sozmen, M. & Pearson, G. R. (1999).** Infection of gnotobiotic calves with *Escherichia coli* O157:H7 strain A84. *Vet Rec* **144**, 466–470.
- Wray, C., McLaren, I. M., Randall, L. P. & Pearson, G. R. (2000).** Natural and experimental infection of normal cattle with *Escherichia coli* O157. *Vet Rec* **147**, 65–68.
- Yokomizo, Y., Watanabe, F., Imada, Y., Inumaru, S., Yanaka, T. & Tsuji, T. (2002).** Mucosal immunoadjuvant activity of the low toxic recombinant of *Escherichia coli* heat-labile enterotoxin produced by *Bacillus brevis* for the bacterial subunit or component vaccine in pigs and cattle. *Vet Immunol Immunopathol* **87**, 291–300.
- Zhao, T., Doyle, M. P., Harmon, B. G., Brown, C. A., Mueller, P. O. & Parks, A. H. (1998).** Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *J Clin Microbiol* **36**, 641–647.