

COLONISATION AND PERSISTENCE OF
Escherichia coli O157:H7 IN THE BOVINE
GASTRO-INTESTINAL TRACT

By

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Acknowledgements

Declaration

I declare that this thesis and the experiments described therein are my own work, except where otherwise indicated. No part of this thesis has been submitted for a degree at this or any other University. All sources of information have been acknowledged by means of reference.

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Abstract

E. coli O157:H7 is a cause of infectious intestinal disease in humans, primarily in developed countries. Although not as prevalent as certain other bacterial enteropathogens, it is of particular concern due to the effect of a secreted virulence factor, shiga toxin (Stx), which causes potentially fatal systemic sequelae. Domestic ruminants, most frequently cattle, are consistently identified as the source of infection and transmission may occur through a variety of routes. The bacterium asymptomatically colonises the gastro-intestinal tracts (GIT) of its ruminant hosts. Adaptation to this niche is responsible for the presence of the organism within the environment. The principal aim during this study was to develop appropriate *in vitro* and *in vivo* systems to examine colonisation mechanisms of *E. coli* O157:H7 in the bovine GIT. An adherence assay on cultured tissue explants was developed to compare different factors involved in *E. coli* O157:H7 adherence. In two separate experiments the contribution of factors involved in intimate attachment, thought to be essential for virulence in humans, was assessed. The ability to intimately attach did not affect the level of *E. coli* O157:H7 adherence to bovine intestinal epithelium *in vitro*. A strain lacking the genes required for intimate attachment however exhibited enhanced adherence to bovine Peyer's patch. The other strains did not exhibit a tropism for any of the tissue types examined.

The most relevant system to assess the behaviour of *E. coli* O157:H7 is within its natural host. Persistent colonisation of weaned calves was achieved for a number of isolates marked by nalidixic acid resistance, including a Stx negative strain that colonised at a similar level and duration to the Stx positive co-strain. At the conclusion of each calf colonisation experiment, those individuals still shedding the organism were examined under necropsy to determine its distribution. The first attempts failed to recover the organism in significant numbers at any site examined despite it being present in ante-mortem faeces. One explanation was that the organism was multiplying primarily in the distal rectum. Further necropsies revealed that the organism was colonising the mucosal surface of the distal 3 cm of the rectum via intimate attachment and confirmed that this phenomenon was indeed typical of persistently colonised calves. This small region contained a high density of lymphoid tissue. Other bacteria are known to have a tropism for follicle-associated

epithelium and it is proposed that *E. coli* O157:H7 possesses an FAE specific factor that mediates its unique distribution within the bovine GIT and is responsible for many aspects of its biology resulting in its importance as a human pathogen.

Common Abbreviations

A/E	Attaching and Effacing
AEEC	Attaching and Effacing <i>E. coli</i>
GB ₃	Globotriaosylceramide
CCE	Calf Challenge Experiment
CFUg ⁻¹	Colony Forming Units per Gram
EHEC	Enterohaemorrhagic <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
Esp	<i>E. coli</i> Secreted Protein
HC	Haemorrhagic Colitis
HUS	Haemolytic Uraemic Syndrome
LEE	Locus of Enterocyte Effacement
IMS	Immunomagnetic Separation
Nal	Nalidixic Acid
PBS	Phosphate Buffered Saline
PCR	Polymerise Chain Reaction
rpm	Revolutions per Minute
STEC	Shigatoxigenic <i>E. coli</i>
TBx	Tryptone x-Glucuronide
P	Positive by Enrichment Culture
N	Negative by Enrichment Culture

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Introduction

1.1 Introduction to *Escherichia coli* (*E. coli*)

E. coli belong to the family *Enterobacteriaceae* a group of 29 genera of Gram negative, non-acid fast, rod-shaped bacteria that share several biochemical and serological characteristics. They are typically oxidase negative, catalase positive and able to reduce nitrate to nitrite (Neidhardt, 1996). Certain specific serotypes are capable of causing severe intestinal or systemic disease, e.g. *Shigella dysenteriae* (dysentery) and *Yersinia pestis* (bubonic plague). Many species have adapted to survive within the diverse environment of the gut as commensals in the gut microbial microflora. The genus *E. coli* is one of the most genetically diverse strains that naturally occur in nature, with a variety of host environments by a range of different mechanisms, some of which have harmful effects on the host. The diversity and regulation of the bacterial factors responsible for survival and pathogenicity have been well characterised in relation to the ultimate goal of developing strategies to prevent or treat some of the more serious disease outcomes of these organisms.

Chapter 1

Introduction

Through the acquisition of resistance factors and progressive evolution, *E. coli* has adapted to survive in a range of vertebrate hosts. The majority of *E. coli* are non-pathogenic and constitute a small proportion of the commensal population within the diet rich mammalian host (USA). The *E. coli* species is a genetically heterogeneous group of organisms, the most extensively studied of which are those that bring about pathological changes within either their primary or incidental hosts and have been classified into several distinct serotypes. Although some pathotypes cause disease at extra-intestinal sites, e.g. UPEC (uropathogenic *E. coli*) in the urinary tract (Sussman and Gally, 1999), the majority cause intestinal disease, typically characterised by diarrhoea (Nislow and Kaper, 1998). The resultant diarrhoea may benefit the organism by enhancing its dissemination or may simply be an incidental effect of factors that have evolved to overcome defences in more hazardous vertebrate hosts. The need to possess various factors in order to overcome host defences, compete with other commensals and survive outside the GIT has led to the evolution

1.1 Introduction to *Escherichia coli* (*E. coli*)

E. coli belong to the family *Enterobacteriaceae* a group of 29 genera of Gram negative, non-acid fast, rod-shaped bacteria that share several biochemical and morphological characteristics. They are typically oxidase negative, catalase positive and able to reduce nitrate to nitrite (Neidhardt, 1996). Certain species/serovars/serotypes are capable of causing severe intestinal or systemic disease, e.g. *Salmonella typhi* (typhoid fever), *Shigella dysenteriae* (dysentery) and *Yersinia pestis* (bubonic plague). Many species have adapted to survive within the enteric environment of vertebrate hosts as components of the commensal microflora. The species *E. coli* is composed of a vast number of genetically diverse strains that collectively have adapted to survive within a variety of host environments by a range of different mechanisms, some of which elicit harmful effects on the host. The function and regulation of the bacterial factors responsible for survival and pathogenesis have been and continue to be studied with the ultimate goal of developing strategies to prevent or treat some of the more serious disease outcomes of these organisms.

1.2 Diarrhoeagenic *E. coli*

Through the acquisition of colonisation factors and progressive evolution, *E. coli* has adapted to survive in a range of vertebrate hosts. The majority of *E. coli* are non-pathogenic and constitute a small proportion of the commensal population within the host gastrointestinal tract (GIT). The *E. coli* species is a genetically heterogeneous group of organisms, the most extensively studied of which are those that bring about pathological changes within either their primary or incidental hosts and have been classified into several distinct pathotypes. Although some pathotypes cause disease at extra-intestinal sites, e.g. UPEC (uropathogenic *E. coli*) in the urinary tract (Sussman and Gally, 1999), the majority cause intestinal disease, typically characterised by diarrhoea (Nataro and Kaper, 1998). The resultant diarrhoea may benefit the organism by enhancing its dissemination or may simply be an incidental effect of factors that have evolved to overcome defences in more immuno-competent hosts. The need to possess certain factors in order to overcome host defences, compete with other commensals and survive outside the GIT has led to the evolution

of many factors present on mobile genetic elements (Syvanen, 1984) such as plasmids or phages. Horizontal transfer of these has resulted in considerable heterogeneity of *E. coli* strains with each possessing a different compliment of colonization/virulence factors. The major pathotypes of *E. coli* and their key virulence determinants are summarized in table 1.1. Common attributes of these bacteria are the ability to colonise the host epithelial surface and to secrete factors (toxins) that are frequently responsible for pathogenesis (Finlay and Falkow, 1997). For example enteropathogenic *E. coli* (EPEC) intimately attach to intestinal epithelium via a well-described mechanism (Frankel et al., 1998) whereas enterotoxigenic *E. coli* (ETEC) attach to enterocytes via fimbriae and elicit their pathogenic effect via heat stable (ST) and/or heat labile (LT) enterotoxins (Nataro and Kaper, 1998).

One pathotype of *E. coli*, related to EPEC, are known as the shiga-toxigenic *E. coli* (STEC, also known as verotoxigenic *E. coli* (VTEC)) due to the presence of shiga-toxin (Stx, also known as verotoxin or verocytotoxin (VT)) (Konowalchuck et al., 1977). Shiga toxin is a secreted product encoded by a bacteriophage present on the chromosome of STEC (Scotland et al., 1983). It is responsible for the serious systemic sequela that may occur in humans following infection with Stx-producing organisms (Karmali et al., 1983). It is also a virulence factor of *Shigella dysenteriae*, a human pathogen closely related to *E. coli*, in which it is the primary virulence factor causing haemorrhagic diarrhoea (dysentery). Details of the molecular biology of Stx are covered in section 1.6.1.3. The Stx bacteriophage has been incorporated into the chromosomes of a variety of *E. coli* serotypes and they are present in a high proportion of faecal samples within host species such as cattle (Jenkins et al., 2002).

Strains from a variety of serotypes have inherited the Stx bacteriophage but only a proportion of these are classified as enterohaemorrhagic *E. coli* (EHEC). The additional requirements for membership of this pathotype are the ability to cause certain disease syndromes in humans and the possession of additional virulence determinants, namely the *LEE* pathogenicity island (locus of enterocyte effacement) and a 60 Mda virulence plasmid (Nataro and Kaper, 1998). There is geographical variation in the incidence and range of serotypes identified as EHEC but certain serogroups, e.g. O157, O26, O111 and O128 are consistently associated with cases of

human haemorrhagic intestinal disease. The most important EHEC serotype world wide, and certainly in the UK and North America, in terms of human disease is O157:H7.

Table 1.1. Gastrointestinal Pathotypes of *E. coli* (Information from Hovland and Kaper, 1998)

Pathotype	Disease Characteristics	Toxins	Adhesions	Adhesin
Enteroblastic <i>E. coli</i> (ETEC)	Watery diarrhoea in young children and naive adults. Also most specific antigen ETEC e.g. K88 in calves.	Heat stable (stabil) heat labile (labile)	Non-stimulate adhesion	Fimbriae (rigid bundles) forming mucus and non-adhesins
Enteropathogenic <i>E. coli</i> (EPEC)	Watery diarrhoea in infants humans and animals	Proteins secreted directly into host cells may affect function	Localized adherence, possibly via fimbriae followed by intimate attachment	Bundle-forming pili (BFP) intimin (FliC)
Enterohaemorrhagic <i>E. coli</i> (EHEC)	Bloody diarrhoea that may progress to systemic disease syndromes	Shiga toxin, Enterohaemorrhagic EAST1	As for EPEC	Intimately adhere and various mucin adhesins
Enterohydrating <i>E. coli</i> (EAEC)	Persistent, mucoid diarrhoea in developing countries	Enterohydrating heat-stable toxin (EAST1), 105 kDa Cytotoxin	Aggregative adherence on HEp-2 cells and formation of biofilm <i>in vivo</i>	Aggregative adherence fimbriae (AAF) and
Enteroinvasive <i>E. coli</i> (EIEC)	Outbreaks of watery diarrhoea	Possible enterotoxin produced by sea	Invasive with similar mechanism to Shiga toxin	PNV fimbriae
Diffusely Adherent <i>E. coli</i> (DAEC)	Watery diarrhoea with higher incidence in older (4-5 years) children	None defined	Non-stimulate adhesion	Fimbriae (F18) ADA1

Table 1.1 Diarrhoeagenic Pathotypes of *E. coli* (information from Nataro and Kaper (Nataro and Kaper, 1998))

Pathotype	Disease Characteristics	Toxins	Adherence Mechanisms	Adhesins
Enterotoxigenic <i>E. coli</i> (ETEC)	Watery diarrhoea in young children and naive adults. Also host specific animal ETEC e.g. K99 in calves	Heat stable and/or heat labile toxins	Non-intimate diffuse adherence	Fimbriae (rigid rods, bundle-forming and fibrillar) and non-fimbrial adhesins
Enteropathogenic <i>E. coli</i> (EPEC)	Watery diarrhoea in infant humans and animals	Proteins secreted directly into host cells may affect function	Localised adherence, initially via fimbriae followed by intimate attachment	Bundle-forming pili and intimin/Tir interaction
Enterohaemorrhagic <i>E. coli</i> (EHEC)	Bloody diarrhoea that may progress to systemic disease syndromes	Shiga toxin, Enterohaemolysin, EAST1	As for EPEC	Intimin/Tir interaction and various putative adhesins
Enteroaggregative <i>E. coli</i> (EAEC)	Persistent, mucoid diarrhoea in developing countries	Enteroaggregative heat-stable toxin (EAST1), 108 KDa Cytotoxin	Aggregative adherence on HEp-2 cells and formation of bacterio-mucus biofilm <i>in vivo</i>	Aggregative adherence fimbriae I and II
Enteroinvasive <i>E. coli</i> (EIEC)	Outbreaks of watery diarrhoea	Possible enterotoxin encoded by <i>sen</i>	Invades with similar mechanism to <i>Shigella</i> spp.	pINV plasmid
Diffusely Adherent <i>E. coli</i> (DAEC)	Watery diarrhoea with higher incidence in older (4-5 years) children	None described	Non-intimate diffuse adherence	Fimbriae (F1845) AIDA-I

1.3 Culture, detection and typing of *E. coli* O157:H7

E. coli are able to rapidly multiply in the presence of oxygen, with a doubling time of 20 minutes in rich media such as Luria Bertani broth (Neidhardt et al., 1974). The ability to grow readily on solid media such as agarose provides a convenient method of isolating, identifying and selecting individual colonies, which greatly facilitates diagnosis. An example of this is MacConkey agar (Balows et al., 1991). The ability to form colonies in the presence of oxygen and bile salts eliminates the majority of commensal strains within a faecal sample (selection) and the pH indicator phenol red identifies colonies as lactose (the carbohydrate source in this media) fermenters. A selective medium for the detection of *E. coli* O157:H7 is CT-SMAC (cefexime-tellurite sorbitol MacConkey) (Zadik et al., 1993). This media makes use of this serotype's characteristics of resistance to the antimicrobials cefixime and tellurite and an inability to ferment sorbitol (March and Ratnam, 1986), which replaces lactose as the carbohydrate source and results in colourless rather than red colonies. *E. coli* O157:H7 differs from the majority of *E. coli* in that it does not ferment the sugars rhamnose (Chapman et al., 1991) or β -glucuronide (Thompson et al., 1990). The latter characteristic is used in MUG (methyl-umbelliferyl-D-glucuronide) containing media to differentiate *E. coli* O157:H7 from other *E. coli* serotypes. To increase the sensitivity of detection pre-enrichment in a variety of selective liquid media has been utilised prior to plating (Table 1.2) with 42°C being the optimal selective temperature. The technique of immunomagnetic separation (IMS) prior to the enrichment step has further increased the sensitivity of detection protocols (Karch et al., 1996). The isolation technique currently favoured by many workers involves broth enrichment, selection by IMS and plating onto sorbitol MacConkey agar containing cefixime and tellurite (Voitoux et al., 2002). Suspect colonies can be confirmed as O157 by the use of relevant antisera and several commercial kits are available that utilise latex conjugated antibodies to O157 (Sowers et al., 1996).

Once isolated and identified, *E. coli* O157:H7 isolates may be further typed using various methods to provide a means of determining clonal relatedness of strains to enable sources of infection to be traced and provide a tool for epidemiological studies. Certain molecular typing techniques e.g. multi-locus enzyme electrophoresis (Whittam et al., 1988), plasmid profiling (Paros et al., 1993) and ribotyping (Martin et al., 1996) are not sufficiently discriminatory since *E. coli* O157:H7 is a highly conserved clone. Reference laboratories make use of the combination of *Stx* gene typing with specific PCR primers and phage typing (Frost et al., 1993). Phage typing is dependant upon susceptibility to lysis induced by a standard set of bacteriophages. This in turn depends upon the expression of surface molecules acting as phage receptors. These are sufficiently variable between strains of *E. coli* O157:H7 to be utilised as a typing technique. Random amplified polymorphic DNA (RAPD) PCR uses arbitrary primers and low stringency PCR to successfully distinguish between *E. coli* O157:H7 isolates (Madico et al., 1995). Digestion of genomic DNA followed by Southern blot hybridisation or analysis by pulsed field gel electrophoresis (PFGE) produce discriminatory gel patterns (Gautom, 1997). The latter is a labour intensive method although in the case of *E. coli* O157:H7 is highly sensitive at detecting strain differences.

1.4 EHEC Mediated Disease

1.4.1 Human EHEC Infection

EHEC cause a spectrum of disease in the human host, the most severe of which are attributed to the systemic activity of *Stx* (Karmali et al., 1983). A certain proportion of infections will be asymptomatic (Ludwig et al., 2002). Some individuals will have a non-bloody diarrhoea lasting a few days which can progress to haemorrhagic colitis, characterised by abdominal pain, haematochezia, and usually a normal body temperature (Riley et al., 1983). Visualisation of the colonic mucosa by colonoscopy reveals erythematous and oedematous mucosa. Abdominal radiographs following a barium enema reveal lesions caused by submucosal oedema that are described as “thumbprinting” lesions. Blood cytology typically reveals a leukocytosis and a thrombocytopenia. Following a prodromal diarrhoeal phase, a proportion of EHEC infections progress to one of the serious manifestations, namely HUS (haemolytic

uraemic syndrome) or TTP (thrombotic thrombocytopenic purpura). Classical HUS (HUS with a prodromal diarrhoeal phase) is characterised by the triad of microangiopathic haemolytic anaemia, thrombocytopenia and acute renal dysfunction in young children. STEC are the most common cause of classical HUS in the western world although other causes include; *Shigella dysenteriae* (in developing countries), some strains of pneumococci and various viruses and drugs. The clinical signs of STEC induced HUS are the result of systemically disseminated Stx (see section 1.6.1.4). Acute renal failure leads to oliguria or anuria and haemolysis exacerbates the anaemia caused by intestinal haemorrhage. Leukocytosis, fever and neurological abnormalities are common findings. TTP is a syndrome that occurs in adults and is not usually preceded by an initial episode of diarrhoea (Pickering et al., 1994). Neurological signs and fever are the typical manifestations.

1.4.2 *E. coli* O157:H7 and Other EHEC and EPEC Associated with Bovine Diarrhoea

Prior to 1982, only one record exists of *E. coli* O157:H7 isolation from cattle. This was from an individual animal in a survey of calves with coli bacillosis in Argentina in 1977 (Orskov et al., 1987). Apart from this record, cattle infected with *E. coli* O157:H7 typically remain asymptomatic and shed bacteria into the environment at levels up to 10^6 organisms per gram of faeces. Infection models of *E. coli* O157:H7 in cattle exist that induce disease (see section 1.8.3.1) but aspects of these do not reflect natural exposure to the organism. The disease reproduced in these models resembles that produced by other *E. coli* strains exhibiting the A/E phenotype (known collectively as AEEC (attaching and effacing *E. coli*)), both naturally occurring and experimentally induced, in that there is extensive colonisation of the intestinal epithelium via A/E lesions accompanied by diarrhoea that is often bloody and mucoid (table 1.3). Almost all these reports involve Stx producing organisms and the possibility exists that Stx is responsible for the intestinal haemorrhage observed. In addition, most of these reports involve very young or pre-weaned animals, suggesting an age related susceptibility to the disease caused by this organism. A variety of serotypes have been associated with bovine diarrhoea and

many of these, as well as several other serotypes, may also asymptotically colonise older, i.e. post-weaned, cattle (Jenkins et al., 2002). *E. coli* O157:H7, despite sharing factors such as the *LEE* and *Stx*, appears to be relatively poor at causing disease within its natural host. It is surprising that other serotypes with a seemingly greater potential to induce disease, at least in cattle, are far less frequently associated with human disease than *E. coli* O157:H7. This indicates that the outcome of infection with these organisms is clearly dependant upon an array of host and bacterial factors.

Table 1.3 Examples of Natural and Experimentally Induced Disease Associated with *E. coli* Serotypes

Serotype	Experimental or Natural	Age of Hosts	Clinical Signs	Sex	Reference
O157	Natural	Calf	Diarrhoea	NO	Wright et al.
O157	Both	16-18 years	Stools with mucoid diarrhoea	Stx 4	Wright et al.
O157	Both	2 days	Large mucoid stools w/ blood	Stx 4	Wright et al.
O157	Both	5 weeks	Bloody and mucoid diarrhoea	Stx 4	Pharmton et al.
O157	Natural	3 weeks	Yellow watery diarrhoea	Stx 4	Pharmton et al.
O157	Experimental	1-11 days	Bloody and mucoid faeces	Stx 1	Wright et al.
O157	Experimental	1-17 days	Bloody and mucoid faeces	Stx 2	Wright et al.
O157	Experimental	15 months	Bloody and mucoid diarrhoea	NO	Wade et al.
O157	Natural	19 months	Bloody and mucoid diarrhoea	Stx 1 and 2	Wright et al.
O157	Experimental	New born	Bloody and mucoid diarrhoea	Stx 4	Wright et al.
O157	Experimental	Weeks	Bloody and mucoid diarrhoea	Stx 1 and 2	Pharmton et al.
O157	Experimental	3-12 months	Bloody diarrhoea	Stx 1	Pharmton et al.
O157	Natural	2 weeks	Mucoid diarrhoea	Stx 1	Wright et al.
O157	Both	2 weeks	Mucoid diarrhoea	Stx 1	Wright et al.
O157	Natural	2 weeks	Bloody diarrhoea	Stx 1	Wright et al.
O157	Experimental	4 and 11 days	Diarrhoea (bloody in 4 day-old calves)	Stx 1	Wright et al.

NO - Not Described
Stx - Shiga toxin

Table 1.3 Examples of Natural and Experimentally Induced Disease Associated with A/E Lesion Forming Strains of *E. coli*

Serotype	Experimental or natural	Age of Hosts	Clinical Signs	Stx Producing	Reference
ND	Natural	Calf	Dysentery	ND	(Moon et al., 1979)
O5:H-	Both	18-21 days	Bloody and mucoid diarrhoea	Stx +	(Hall et al., 1985)
O5:H-	Both	2 days	Loose mucoid faeces +/- blood	Stx +	(Moxley and Francis, 1986)
O111:NM	Both	5 weeks	Bloody and mucoid diarrhoea	Stx +	(Schoonderwoerd et al., 1988)
O80:NM	Natural	3 weeks	Yellow watery diarrhoea	Stx -	(Pearson et al., 1989)
O26:H11	Experimental	1-17 days	Bloody and mucoid faeces	Stx 1	(Wray et al., 1989)
O8:H9	Experimental	1-17 days	Bloody and mucoid faeces	Stx 2	(Wray et al., 1989)
O15	Natural	19 months	Bloody and mucoid diarrhoea	ND	(Wada et al., 1994)
O157:H7	Experimental	New born	Bloody and mucoid diarrhoea	Stx 1 and 2	(Dean-Nystrom et al., 1997)
O157:H7	Experimental	Weaned	Bloody and mucoid diarrhoea	Stx +	(Dean-Nystrom et al., 1999)
O26	Natural	8-12 months	Bloody diarrhoea	Stx 1 and 2	(Pearson et al., 1999)
O118:H16	Both	2 weeks	Non-bloody diarrhoea	Stx 1	(Stordeur et al., 2000)
O26:H11	Natural	2 weeks	Bloody diarrhoea	Stx1	(Gunning et al., 2001)
O111:H-	Experimental	4 and 11 days	Diarrhoea (bloody in 4 day-old calves)	Stx1	(Stevens et al., 2002)

ND – Not Described

Stx – Shiga toxin

1.5 The Source and Transmission of *E. coli* O157:H7

1.5.1 Cattle as Reservoirs of *E. coli* O157:H7

Although many cases of EHEC infection are sporadic, indeed in the UK these make up the majority of cases, the source of infection is more frequently traced during outbreaks. The importance of cattle as a reservoir for *E. coli* O157 became evident when several outbreaks were associated with the consumption of undercooked beef and other bovine products such as unpasteurised milk (Borczyk et al., 1987; Riley et al., 1983). Cattle are now considered to be the principal source of *E. coli* O157 that cause human disease and transmission may occur through a variety of routes. In addition to the contamination of meat and dairy products, bovine slurry can contaminate drinking water (Akashi et al., 1994; Licence et al., 2001; Yarze and Chase, 2000) and crops intended for human consumption. Various outbreaks have been associated with vegetable products, such as radish (Fukushima et al., 1999) and apple cider (Besser et al., 1993), presumably following contamination with animal slurry. Transmission can also occur following direct contact with shedding animals (Chapman, 2000) and PFGE typing has confirmed the direct transmission from cattle to humans (Louie et al., 1999).

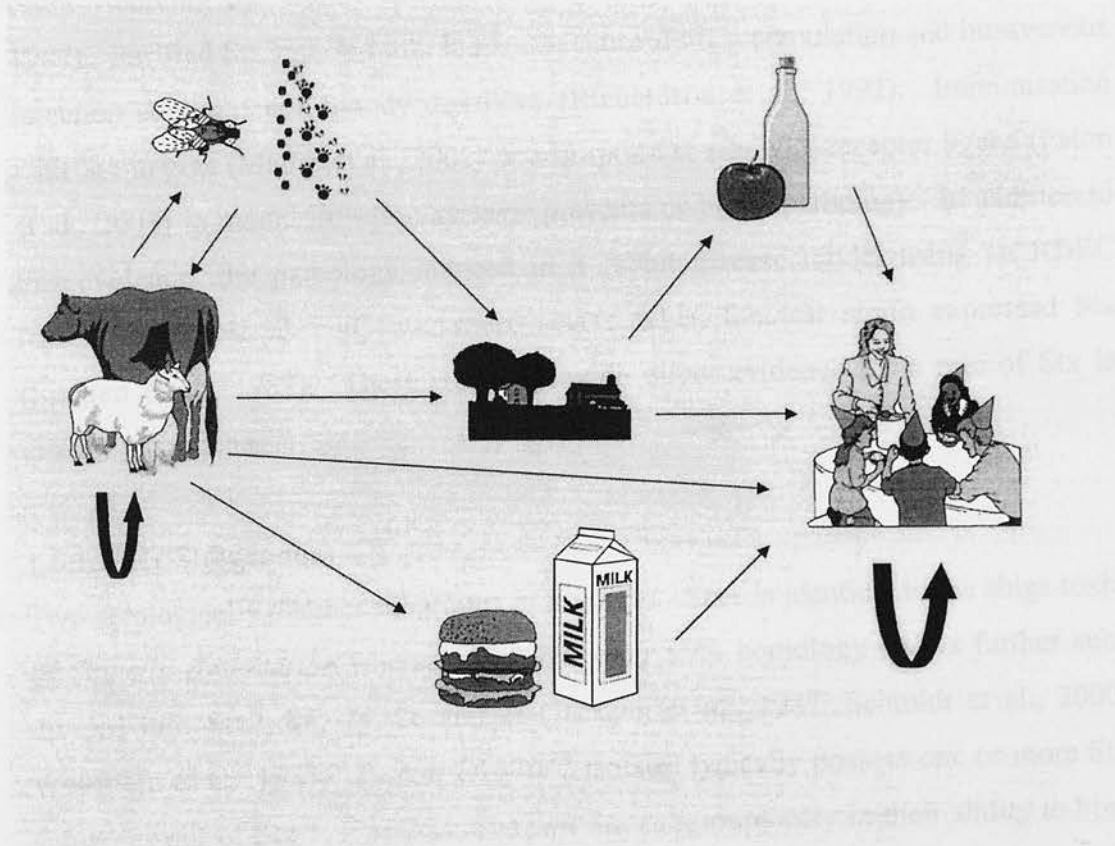
The prevalence of *E. coli* O157:H7 in cattle has been determined by several field studies in slaughterhouses and farms. Estimates of prevalence vary according to the sensitivity of the detection technique used with the trend towards the use of IMS leading to higher estimates of prevalence in more recent studies. On farm prevalence has also been investigated and typical rates are 1.8% in Japan (Miyao et al., 1998), 1.9% in Australia (Cobbold and Desmarchelier, 2000) 1.5% Brazil (Cerqueira et al., 1999) and 0 to 7.4% in the U.S.A. (Faith et al., 1996; Hancock et al., 1997; Laegreid et al., 1999; Wells et al., 1991). Two prevalence studies, in Scotland (Synge and Paiba, 2000) and England and Wales (Paiba et al., 2002) found much higher prevalence rates of 8.6% and 4.7% respectively. A survey of *E. coli* O157:H7 (Elder et al., 2000) in cattle in slaughter-houses in the U.S.A. found the prevalence to be 28% overall.

1.5.2 Other Species as Reservoirs of *E. coli* O157:H7

In addition to cattle, other ruminant species, in particular sheep and deer have been identified as the source of human *E. coli* O157:H7 outbreaks. *E. coli* O157:H7 was recovered from 2.9% of lamb products compared with 1.1% of beef products in a survey of butcher's shops (Chapman et al., 2000). Although the on-farm prevalence in sheep has not been as studied to the same extent, it is thought to be much lower than in cattle, although one study (Kudva et al., 1996) reported a transient prevalence of 31%. A notable outbreak attributed to sheep occurred in a Boy Scout camp at which some of the Scouts admitted to having sheep dung fights (Ogden et al., 2002). Unpasteurized goat milk (*Capra hircus*) has been traced as the source of a human outbreak of *E. coli* O157:H7 infection (Bielaszewska et al., 1997). The outbreak attributed to un-pasteurised apple cider (Besser et al., 1993) was traced to an orchard where wild deer stools were present, although *E. coli* O157:H7 could not be detected. Additional evidence that deer can be reservoir host comes from a study in which white-tailed deer (*Odocoileus virginianus*) were challenged with 10^9 CFU *E. coli* O157:H7 and observed a similar shedding pattern and intestinal recovery to those reported for sheep and cattle (Fischer et al., 2001). However the organism was not consistently recovered from wild deer stools in field studies. Pigs have been suggested to be a potential reservoir of *E. coli* O157:H7 (Nakazawa et al., 1999) although no outbreaks have been traced to porcine products. Other than one outbreak attributed to rabbits (Pritchard et al., 2001), there is little evidence that non-ruminant hosts are important in the maintenance of *E. coli* O157:H7 within the farm environment or its transmission to humans. It has been shown that *E. coli* O157:H7 can multiply within house-flies (*Musca domestica*), an organism which could efficiently disseminate the organism throughout the environment (Sasaki et al., 2000). There is also evidence that humans can be asymptomatic carriers of this organism from an outbreak in a hospital linked nursery in which several kitchen staff were sero-positive for O157 LPS despite having no signs of infection (Cheasty et al., 1998).

Figure 1.1 Flow Chart of *E. coli* O157:H7 Transmission

E. coli O157:H7 has been reported to infect humans via a range of transmission routes, all of them dependant upon the organism being shed within ruminant faeces which then contaminates its environment, food, water or is transmitted by other vectors. Human to human transmission has also been documented.



in and destroy a human microvascular cell line (Jacewicz et al., 1989). Although Stx1 has a 30-fold higher affinity for the cells than Stx2, the latter is more toxic in equivalent amounts. This is consistent with the higher proportion of Stx2 carriage with HEC and HUS.

1.6.1.3 Mechanism of Action

Step 1: ADP, major virulence binding subunit (B) surrounding a catalytic subunit (A) (Neau and Kaper, 1996). Stx binds via the B subunit to the glycolipid (GD, galactose- α -1,3-galactosylceramide) on susceptible cells (Lingwood, 1996) and the A subunit is

1.6 Colonisation and Virulence Factors of *E. coli* O157:H7

1.6.1 Shiga-Toxin

1.6.1.1 Stx Mediated Disease

Several distinct syndromes are proposed to be mediated by Stx: (i) diarrhoea, as a result of compromised intestinal epithelium integrity; (ii) haemorrhagic colitis, due to damage of the endothelium of intestinal mucosal vasculature with alterations in haemostasis and (iii) a systemic phase with disseminated effects on haemostasis often involving the kidney and the central nervous system (O'Brien and Holmes, 1987). Purified Stx injected into loops can cause fluid accumulation and intravenous injection causes a non-bloody diarrhoea (Richardson et al., 1992). Immunisation with Stx in pigs (Matise et al., 2001) or adsorption *in situ* with receptor ligand (Paton et al., 2001) in model infection systems prevents or limits pathology. In addition to this evidence, the pathology induced in a rabbit disease model using an RDEC (rabbit diarrhoeic *E. coli*) was more severe when the test strain expressed Stx (Sjogren et al., 1987). These studies provide direct evidence for a role of Stx in disease pathogenesis.

1.6.1.2 Stx Subgroups

Two serologically distinct subgroups of Stx exist. Stx1 is identical to the shiga toxin of *Shigella dysenteriae* whereas Stx2 has only 56% homology and is further subdivided into Stx2, 2c, 2d, 2e and 2f (Jackson et al., 1987; Schmidt et al., 2000; Weinstein et al., 1988). *E. coli* O157:H7 isolates typically possess one or more Stx sub-types out of Stx 1, 2 or 2c. The two Stx subgroups vary in their ability to bind to and destroy a human microvascular cell line (Jacewicz et al., 1999). Although Stx1 has a 50 fold higher affinity for the cells than Stx2, the latter is more toxic in equivalent amounts. This is consistent with the higher association of Stx2 carriage with HC and HUS.

1.6.1.3 Mechanism of Action

Stxs are AB₅ toxins with five binding subunits (B) surrounding a catalytic subunit (A) (Sears and Kaper, 1996). Stx binds via the B subunits to the glycolipid GB₃ (globotriaocylceramide) on susceptible cells (Lingwood, 1996) and the A subunit is

endocytosed, transported within vesicles and proteolytically nicked by the protease furin to generate the catalytically active A₁ subunit. In sensitive cells this is transported to the golgi apparatus and ultimately to the endoplasmic reticulum (Sandvig et al., 1992) where its N-glycosidase activity removes an adenine residue from the 28S sub-unit of 60S ribosomal RNA (Endo et al., 1988). The resultant inhibition of protein synthesis is thought to be responsible for cell death although some of its effects may be more subtle, such as the induction of apoptosis and the up-regulation and release of certain cytokines (Proulx et al., 1998; Thorpe et al., 1999).

The genes encoding Stx are located on lytic phages within the bacterial chromosome (O'Brien et al., 1984). Optimal expression of Stx occurs during the lytic phage phase that is in turn induced by the bacterial SOS response (Fuchs et al., 1999). An important consequence of this is that the use of certain anti-bacterial agents (fluorquinolones) for HC patients is not recommended since the resultant DNA damage induces the SOS response (Matsushiro et al., 1999).

1.6.1.4 Pathogenesis of Stx Disease

Although the bacterial pathogenesis of neither HC nor HUS have been fully elucidated, a prerequisite is presumably bacterial multiplication within the intestine with a proportion of bacteria undergoing phage mediated lysis to release Stx. It is likely that during the disease process, mucosal adherence, probably by intimate attachment (1.6.2) occurs and this together with other putative virulence factors may compromise the mucosal surface and induce inflammation. There is also evidence that Stx acts directly on the epithelium to induce pathological changes (O'Brien and Holmes, 1987). Culture supernatant of *E. coli* O157:H7 and other EHEC serotypes provoked fluid accumulation in rabbit ileal loops, the severity of which correlated with the level of Stx (Ferreira et al., 1997). The small proportion of bacteria that undergo phage mediated lysis and release Stx may benefit the surviving population by possible effects on the host immune response or by promoting intestinal haemorrhage to provide a source of iron, when this occurs.

Although Stx has been shown *in vitro* to cross intact epithelial barriers (Philpott et al., 1997), the activity of other bacterial factors to induce inflammation and compromise the integrity of the intestinal epithelium may enhance absorption of

Stx. Once it has crossed the epithelial barrier, Stx targets GB₃ expressing endothelium of the mucosal vasculature. Alterations to the haemostatic pathways result in a thrombotic microangiopathy (Richardson et al., 1988). In conjunction with Stx toxicity, inflammatory responses are important during STEC infection in humans (Eisenhauer et al., 2001; Foster et al., 2000; Ohmi et al., 1998). In HUS patients pro-inflammatory cytokines (IL-1 β , IL-8, TNF α) are elevated, which sensitise endothelial cells to the action of Stx by inducing further GB₃ expression (Richardson et al., 1988; Vandekar et al., 1992). The progression of human STEC infection thus involves both toxic and immunopathological elements. The pro-inflammatory cytokine IL-8 is upregulated by Stx *in vitro* (Thorpe et al., 1999) and this mediates chemo-attraction of polymorphonucleocytes (PMN) such as neutrophils. One hypothesis for the dissemination of Stx is that it is transported through the circulation on the surface of PMNs (te Loo et al., 2000), although other mechanisms have been suggested, including adherence to thrombocytes (Cooling et al., 1998).

Once systemically disseminated the resultant disease depends upon the distribution of GB₃ receptors within the host. In human infants these are most significantly present on the vascular endothelium of the kidney glomeruli (Boyd and Lingwood, 1989). The subsequent renal pathology results in HUS which is potentially fatal and more likely to occur in young children than other age groups of humans. A potentially fatal systemic consequence of Stx absorption in humans is thrombotic thrombocytopenic purpura which is a result of Stx targeting CNS (Ruggenti and Remuzzi, 1998).

1.6.1.5 Stx in Cattle

STEC have a high prevalence within cattle, e.g. 24% of Japanese cattle with faeces PCR positive for Stx. There is no evidence that Stx is toxic to ruminant hosts and certainly in healthy weaned cattle *E. coli* O157:H7 is non pathogenic. One explanation for the lack of virulence associated with Stx in cattle may be the presence of GB₃ receptors on cells in the basal crypts (Hoey et al., 2002). Stx is taken up by these cells and processed to lysosomes and destroyed, in contrast to Stx sensitive cells in which it is transported to the endoplasmic reticulum where it

inhibits protein synthesis (Hoey et al., 2003). This may result in the adsorption of Stx, limiting systemic dissemination. In addition the distribution and isoforms of GB₃ receptors in bovine organs may render them resistant to any pathological sequela.

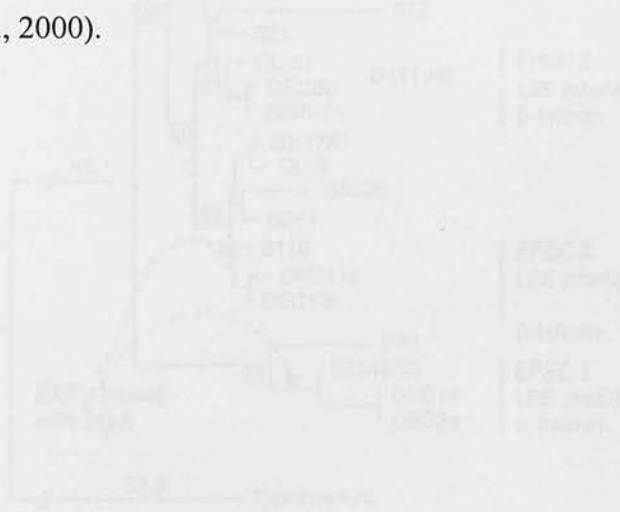
Despite the absence of traditional toxicity it is possible that Stx is acting as a survival or colonisation factor within the bovine GIT environment. This may be a result of immunomodulatory activities that could theoretically down-regulate immune responses that would otherwise shorten the duration of colonisation within an individual or prevent subsequent re-colonisation. Interleukin-8 (IL-8) is a pro-inflammatory cytokine that is chemo-attractant for neutrophils and Stx has been shown to up-regulate expression of IL-8 in human cell lines (Thorpe et al., 1999). In contrast IL-8 is down regulated in bovine primary colonocytes (Hoey, submitted for publication) providing an additional explanation for the lack of pathology and a mechanism that may facilitate colonisation of this host. Alternatively, the prevalence of the Stx genes may simply result from their location within bacteriophages. These toxin-converting bacteriophages may disseminate Stx between *E. coli* strains through horizontal transfer and possess mechanisms to prevent their own loss from the chromosome. The sophistication and subtlety of Stxs however, suggests that they originally evolved within a bacterial species to mediate a survival or colonisation advantage thus enhancing their own persistence and dissemination within the environment of a eukaryote host.

1.6.2 The Locus of Enterocyte Effacement

1.6.2.1 Background

EHEC strains have evolved from different EPEC lineages through independent acquisition of Stx encoding phages (Reid et al., 2000). Phylogenetic analysis has divided both EPEC and EHEC into two clonal groups, clones 1 and 2 (fig. 1.2). The common virulence determinant of EPEC and EHEC strains is the Locus of Enterocyte Effacement (*LEE*) pathogenicity island, a 35.5 Kb chromosomally located region comprising over 40 open reading frames that collectively mediate the ability to form intimate attachment and the associated attaching and effacing lesions (A/E) on the apical surface of host intestinal epithelium (McDaniel and Kaper, 1997). The

LEE of EPEC but not *E. coli* O157:H7, is able to confer the A/E phenotype on *E. coli* K12 (Elliott et al., 1999), suggesting that additional non-*LEE* encoded factors are necessary for either the function or regulation of *LEE* proteins in this serotype. The contribution of the A/E phenotype to disease caused by both EPEC and EHEC in various host species has been confirmed by the deletion of components of the *LEE* including a study of a *LEE* deleted EPEC in humans (Donnenberg et al., 1993). Although a similar study has not been repeated for EHEC in humans, convalescent EHEC patients possess antibodies to many *LEE* encoded factors and *LEE* negative STEC causing HC or HUS are very rare, suggesting a role for A/E in human disease (Li et al., 2000).



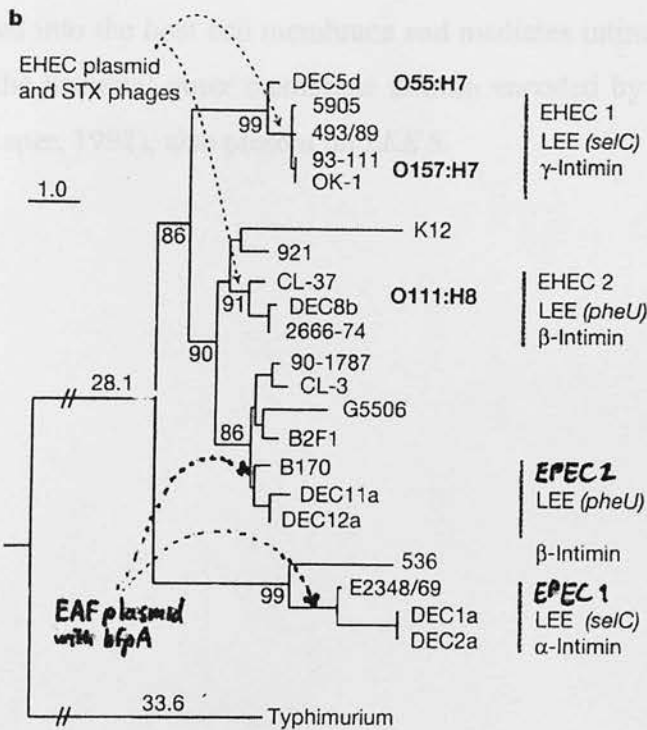
From Reid et al. (2000)

1.5.2.3 Mechanism of A/E Lesion Formation

The majority of genes necessary for A/E lesion formation are present on the *LEE* arranged in 5 polycistronic operons (*LEE* 1 to 5, fig. 1.4). *LEE* 1 to 3 possess genes encoding a type III secretion system, by definition a contact-dependent mechanism (Hueck, 1998) in which proteins are secreted across the bacterial membrane and then transferred directly into the host cell. Crucial to the transfer of proteins into the host cell are some of the products of the genes on *LEE* 4, namely EspA (a cell-associated protein A) that forms hollow filaments anchoring the bacterial cell with the host cell and EspB and D which form a pore within the host cell membrane and interface with the EspA filament (fig. 1.5) (Frankel et al., 1995). Expression of *LEE* 4 proteins amongst *E. coli* O157:H7 isolates varies considerably under certain

Figure 1.2 Phylogenetic tree of EPEC and EHEC Families

This illustrates the relationship between different lineages of EPEC and EHEC and also the importance that horizontal transfer of mobile genetic elements has on the evolution of pathogenic strains.



From Reid et al. (2000)

1.6.2.2 Mechanism of A/E Lesion Formation

The majority of genes necessary for A/E lesion formation are present on the *LEE* arranged in 5 polycistronic operons (*LEE* 1 to 5, fig. 1.4). *LEE* 1 to 3 possess genes encoding a type III secretion system, by definition a contact-dependant mechanism (Hueck, 1998) in which proteins are secreted across the bacterial membranes and then transferred directly into the host cell. Crucial to the transfer of proteins into the host cell are some of the products of the genes on *LEE* 4, namely EspA (*E. coli* secreted protein A) that forms hollow filaments connecting the bacterial cell with the host cell and EspB and D which form a pore within the host cell membrane and interface with the EspA filament (fig 1.5) (Frankel et al., 1998). Expression of *LEE* 4 proteins amongst *E. coli* O157:H7 isolates varies considerably under certain *in*

vitro conditions and it has been suggested that human isolates are more likely to be high expressors than bovine isolates (McNally et al., 2001). Proteins that are transferred to the host cell via the type III secretion system include the pro-apoptotic molecule EspF (Crane et al., 2001), MAP (mitochondrial associated protein) (Kenny et al., 2002) and Tir (translocated intimin receptor) (Kenny et al., 1997). The latter is inserted into the host cell membrane and mediates intimate attachment by interacting with the bacterial outer membrane intimin encoded by *eaeA* (Jerse et al., 1990; Yu and Kaper, 1992), also present on *LEE 5*.

Figure 1.3. Arrangement of genes on the *LEE* of *E. coli* O157:H7

Open reading frames are indicated with their products as shown in Fig. 1.4.



Figure 1.3 Arrangement of Genes on the LEE of *E. coli* O157:H7

Open reading frames are colour-coded with their products as shown in Fig. 1.4.

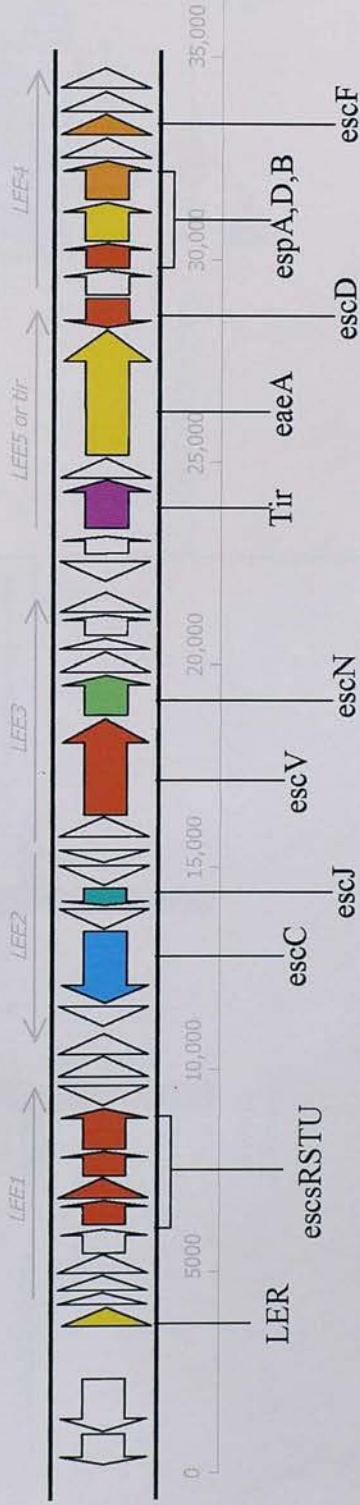


Figure 1.4 Type III Secretion and A/E Lesion Formation

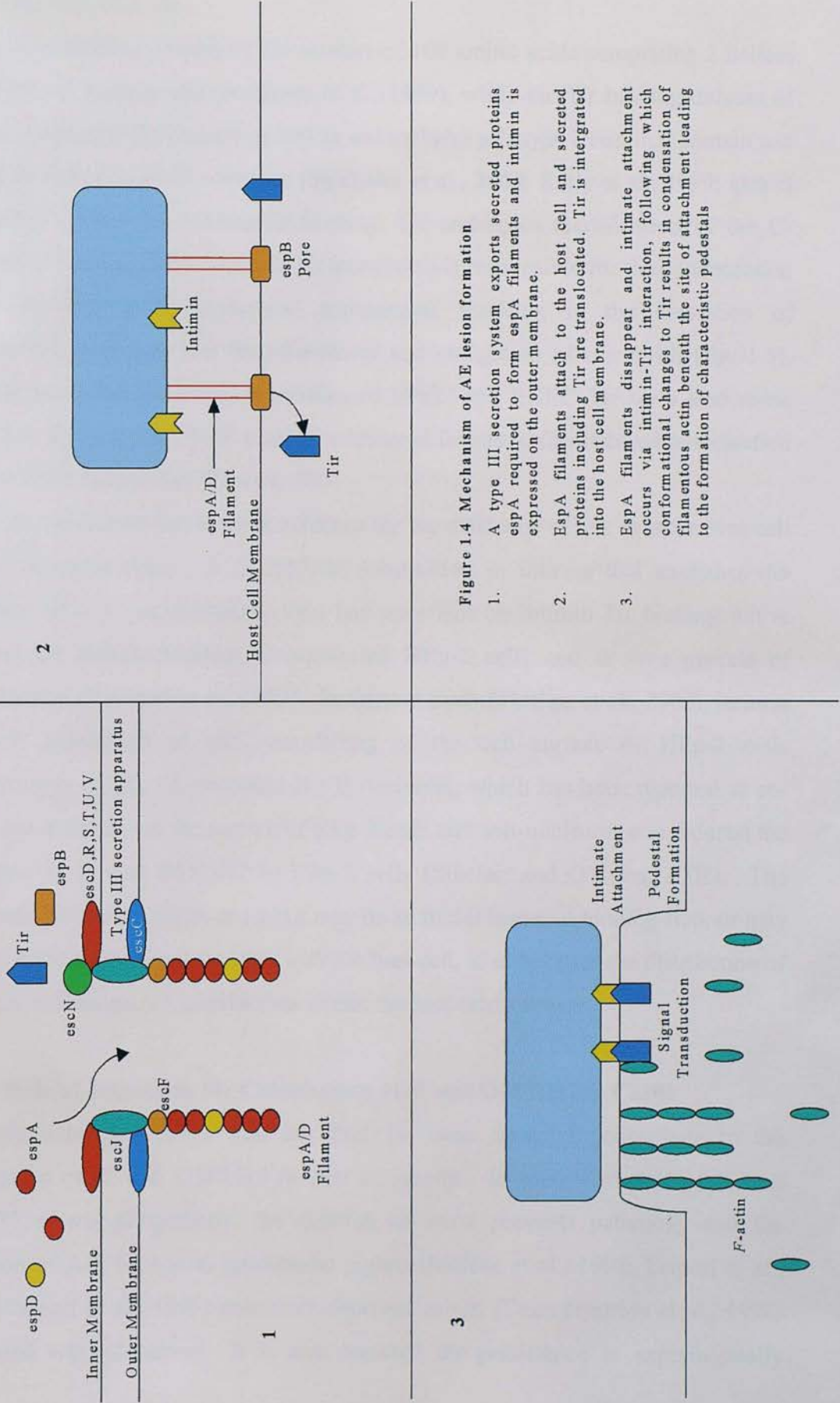


Figure 1.4 Mechanism of A/E lesion formation

1. A type III secretion system exports secreted proteins espA required to form espA filaments and intimin is expressed on the outer membrane.
2. EspA filaments attach to the host cell and secreted proteins including Tir are translocated. Tir is integrated into the host cell membrane.
3. EspA filaments disappear and intimate attachment occurs via intimin-Tir interaction, following which conformational changes to Tir results in condensation of filamentous actin beneath the site of attachment leading to the formation of characteristic pedestals

1.6.2.3 Intimin and Tir

The intimin binding domain of Tir consists of 109 amino acids comprising 2 helices joined by a β hairpin turn (de Grado et al., 1999), while the Tir binding domain of intimin consists of the c-terminal 190 aa and includes a C-type lectin like domain and part of an immunoglobulin domain (Batchelor et al., 2000; Kelly et al., 1999; Luo et al., 2000). Following intimin/Tir binding, Tir undergoes modification of the C-terminal 170 amino acids. Intimin-Tir interaction is responsible for the condensation of several host cell cytoskeletal components resulting in the formation of characteristic pedestals and the effacement and elongation of microvilli (fig. 1.5). Differences in the modification profiles of EPEC and EHEC Tir have lead some workers to suggest that EHEC produce additional factors to facilitate actin nucleation (Kenny, 2001; Kenny and Warawa, 2001).

In addition to Tir, there is evidence for the existence of one or more host cell intimin receptors (Hir). A Cys937Ala substitution in intimin that abolishes the formation of a 76 aa disulphide loop has no effect on intimin-Tir binding but is essential for intimin binding to uninfected HEp-2 cells and *in vivo* models of pathogenicity (Hartland et al., 1999). In another study (Phillips et al., 2000), intimin mediated attachment to and remodelling of the cell surface of HEp-2 cells independently of Tir. A proposed Hir is nucleolin, which has been reported to co-localise to intimin γ on the surface of HEp-2 cells and anti-nucleolin sera reduced the adherence of *E. coli* O157:H7 to HEp-2 cells (Sinclair and O'Brien, 2002). The interaction between intimin and a Hir may be an initial bacterial binding step, or may mediate additional signalling steps with the host cell, in either case the distribution of Hir could determine A/E distribution within the host environment.

1.6.2.4 Role of Intimin in the Colonisation of *E. coli* O157:H7 in Cattle

The only adherence factor that has thus far been shown to contribute to the colonisation of *E. coli* O157:H7 *in vivo* is intimin. In animal models of *E. coli* O157:H7 enteropathogenicity, the deletion of *eaeA* prevents pathology and the formation of A/E lesions in gnotobiotic piglets (McKee et al., 1995; Tzipori et al., 1995) (Tzipori et al., 1995) colostrum-deprived calves (Dean-Nystrom et al., 1998) and fasted weaned calves. It is also required for persistence in experimentally

challenged weaned calves (Cornick et al., 2002). However *E. coli* O157:H7 is not considered to cause disease in naturally colonised cattle and intimately attached bacteria have not been detected in such animals. The faecal concentration of *E. coli* O157:H7 in the studies by Dean-Nystrom was much higher than those typically found in naturally colonised cattle. This could increase the density of intimately attached bacteria above the detection threshold (Dean-Nystrom et al., 1999) to a level where they are both detectable histologically and contribute to pathology. Therefore for *E. coli* O157:H7 there appears to be a correlation between intimin mediated intimate attachment and pathology in the animal models examined. It is possible that in healthy weaned cattle *E. coli* O157:H7, and possibly other AEEC, form A/E lesions at densities below a detectable threshold during sub-clinical infection, and that this interaction is an essential step in the colonisation of this host.

At least five distinct intimin types have been described based on serological and sequence analysis (table 1.4) (AduBobie et al., 1998; Oswald et al., 2000). Intimin α is possessed mainly by EPEC clone 1 that are mostly human pathogens, intimin β is possessed by EPEC clone 2 and EHEC clone 2, intimin γ is possessed by *E. coli* O157:H7 and its proposed progenitor serotype O55:H7, intimin δ is similar to intimin β and is possessed only by EPEC O86:H34 (AduBobie et al., 1998) and intimin ϵ is possessed by various STEC including O103:H2 (Oswald et al., 2000).

Table 1.4 Significant AEEC Strains and Intimin Types

A selection of some of the more important serotypes of AEEC showing the association of intimin type with the ability to cause intestinal disease within certain hosts.

Strain	Intimin Type	Stx	Disease Susceptible Hosts
O127:H6	α	-	human
O55:H6	α	-	human
O118:H16	β	+	calves
O111:H2	β	+	calves/humans
O111:H-	β	+	humans
O26:H11	β	+	calves/humans
O26:H-	β	+	calves/humans
O86:H34	δ	-	humans
O103:H2	ϵ	+	calves/humans
O55:H7	γ	-	humans
O55:H-	γ	-	humans
O157:H7	γ	+	humans

There is evidence that intimin type contributes to host and tissue tropism, for example in gnotobiotic piglets (Tzipori et al., 1995). Using human IVOC the tissue specificity of EPEC (throughout the small intestine) could be changed to that of EHEC (restricted to follicle associated epithelium), by deleting *eaeA* and complementing with intimin γ from *E. coli* O157:H7 (Phillips and Frankel, 2000) and *vice versa* (Fitzhenry et al., 2002).

On bovine IVOC, *E. coli* O157:H7 appears to have a more ubiquitous tissue tropism with A/E lesions demonstrated on large intestinal (Baehler and Moxley, 2000) and Peyer's patch epithelium (Phillips et al., 2000). Studies of bovine AEEC isolates demonstrate a different distribution of intimin types in diarrhoeic calves and healthy adults. Adult isolates were more likely to possess intimin γ whereas diarrhoeic calf isolates mostly possessed intimin β . However this variation in pathotype may not have been mediated by intimin type alone since different Tir,

EspA and EspB sub-types were associated with certain intimin types (China et al., 1999; China et al., 1999). Reports of calf diarrhoea, both natural and experimental, where A/E lesions have been demonstrated invariably involve strains with non- γ intimin types such as β (O26 (Pearson et al., 1999), O80 (Wray et al., 1989) and O118 (Stordeur et al., 2000)), whereas *E. coli* O157:H7 has only been reported to cause disease in neonatal calves (Dean-Nystrom et al., 1997) or weaned calves following a period of fasting (Dean-Nystrom et al., 1999). In many countries *E. coli* O157:H7 is the EHEC serotype most commonly attributed to human disease, although non-intimin γ expressing EHEC are also important (Bettelheim, 2000).

1.6.3 Other Putative Chromosomally Located Virulence/Colonisation Factors of *E. coli* O157:H7

1. A factor present in *E. coli* O111, Efa1 (*E. coli* factor for adherence), contributes to the adherence of the organism to Chinese hamster ovary (CHO) cells (Nicholls et al., 2000) and appears to be an essential colonisation factor in cattle (Stevens et al., 2002). A truncated version of the *efa1* gene is also present on the chromosome of *E. coli* O157, in which it is termed *lifA*. A mutation upstream of the *lifA* gene leads to a reduced adhesion to Caco-2 cells (Tatsuno et al., 2000) although this may be due to the possible role of this protein in the secretion of *LEE* proteins.
2. The heat stable enterotoxin EAST1 is found in EAEC where it is important virulence factor (Savarino et al., 1993) and the *astA* gene is found in various EHEC strains including *E. coli* O157:H7 (Savarino et al., 1996), although its significance in these is unknown.
3. IHA (iron regulating factor homologue adhesin) is a gene identified by random mutagenesis to mediate adherence to HeLa and MDBK cells (Tarr et al., 2000).
4. The complete genome sequences of *E. coli* O157:H7 have identified a type III secretion system similar to the *inv/spa* system of *Salmonella* spp (Perna et al., 2001).
5. Two copies of a gene (*cah*) that encode a calcium-binding autotransporter protein may play a role in biofilm formation. (Torres et al., 2002).

1.6.4 The 60 MDa Plasmid of *E. coli* O157:H7 (pO157)

E. coli O157:H7 possesses a 60 MDa plasmid which early workers demonstrated to enhance adherence of the organism to Henle 407 cells (Toth et al., 1990). A plasmid-cured derivative did not colonise a mouse colonisation model as effectively as its parent strain (Wadolowski et al., 1990) but the plasmid did not contribute to the observed pathology in a piglet (Tzipori et al., 1987) disease model. It was initially thought that this plasmid contains a fimbrial gene cluster (Karch et al., 1987) however subsequent sequencing of pO157 revealed that it contains no recognisable fimbrial operon (Burland et al., 1998). Amongst the genes present on pO157 identified as potential virulence determinants are the enterohaemolysin operon (Bauer and Welch, 1996), *EspP* which encodes a SPATE (serine protease autotransporter toxin of *Enterobacteriaceae*) toxin (Brunder et al., 1997) and *toxB* which has homology to *lifA* and contains some motifs in common with the large clostridial toxins *toxA* and *toxB* of *Clostridium difficile* (Burland et al., 1998).

1.6.4.1 Enterohaemolysin (Ehx)

Weak zones of haemolysis can be observed on blood agar containing washed erythrocytes under *E. coli* O157:H7 colonies after 18 to 24 hours (Beutin et al., 1989). Ehx is present and highly conserved amongst STEC strains suggesting that it contributes to survival in some way (Boerlin et al., 1999), however there is conflicting evidence regarding its contribution to the development of HC or HUS (Boerlin et al., 1999; Schmidt and Karch, 1996). Ehx has been shown to be active on human sheep erythrocytes and bovine lymphoma cells (Bauer and Welch, 1996; Schmidt et al., 1996 and Chart et al., 1998). Ehx is produced at very low levels in *E. coli* O157:H7 and this is thought to be due to a defective transporter system (Schmidt et al., 1995), however expression of Ehx has been shown to be optimal *in vitro* under conditions of low oxygen tension (Chart et al., 1998). Expression of the structural toxin gene *in vivo* has been demonstrated indirectly through the detection of Ehx specific antibody in bovine colostrum (Lissner et al., 1996).

1.6.4.2 EspP

The *EspP* (*E. coli* secreted protein P) gene of *E. coli* O157:H7 is a member of the SPATE family of toxins and is one of the most abundant proteins secreted during growth in LB medium (Brunner et al., 1997; Ebel et al., 1996). The function of EspP is poorly characterised although it has been shown to cleave the human coagulation factor V protein. Antibodies to EspP have been found in the serum of convalescent human patients from *E. coli* O157:H7 infection, indicating that the protein is produced during infection of humans.

1.6.4.2 toxB

The sequence of the *toxB* gene shows N-terminal homology to the *toxA* and *toxB* proteins of *C. difficile* and contains a conserved region essential for glycosyltransferase activity in the clostridial toxins (Burland et al., 1998). Deletion of pO157 reduced adherence to Caco-2 cells that was complemented with the region of pO157 containing *toxB* (Tatsuno et al., 2001). However an indirect mechanism for this activity is proposed by the observation that a mutation in *toxB* leads to a four-fold decrease in *LEE4* protein secretion levels. A *toxB* homologue is present in non-O157 EHEC strains such as *E. coli* O111, termed Efa1, that appears to contribute to initial adherence of the organism to CHO cells (Nicholls et al., 2000). It has been identified as an essential factor in experimental colonisation of cattle by *E. coli* O111 (Stevens et al., 2002) although this may be an indirect consequence of its possible role in the *LEE* type III secretion system (Tatsuno et al., 2001)

1.7 Bacterial Adherence

1.7.1 Bacterial Adherence to Intestinal Mucosa

Any bacterial tropism for a particular region or cell type of the GIT requires either an ability to compete for nutrients within the highly specialised commensal microbiota and/or a specific adherence or invasion mechanism to combat the constant flow of digesta (Finlay and Falkow, 1997). Adherence is a colonisation strategy common to a range of pathogenic bacteria and a selection of some of the better-characterised adhesins are summarized in table 1.5. Some adhesins mediate tropism for extensive but distinct regions of the GIT, for example K88 and K99 fimbriae of ETEC to the small intestine (Nagy and Fekete, 1999). The presence of an adhesin may be

detected by the distribution of pathological lesions observed *in vivo*. For example an EPEC strain (of serotype O111) was demonstrated to cause diarrhoea in human volunteers when injected into the duodenum but not the large intestine (Tesh and O'Brien, 1992). Coliforms are only present, if at all, in small numbers within the small intestine, implying an adherence factor (or factors) that mediates a tropism for small intestinal epithelium. Since bacterial adherence factors must, by their very nature, be expressed on the surface of the outer membrane, they are exposed to the adaptive arm of their host's immune system. Adhesin specific antibodies, when present in the mucous layer, have the potential to physically block the interaction necessary for adherence or to opsonise bacteria for killing by other immune mechanisms e.g. complement or phagocytosis. Adhesins are therefore effective vaccine targets in the prevention of intestinal disease (Mouricout, 1991).

Table 1.5 Examples of bacterial Adhesins (Med)

A selection of bacterial-host relationships in which the molecular basis has been defined.

Pathogen	Host Tissue	Adhesin	Receptor
<i>Shigella flexneri</i>	Intestinal epithelia	Protein F	Fibronectin
<i>Enterococcus faecalis</i>	GI epithelium	Mannose adhesin plus	Multi. (GMIAs) chondroitin
<i>Neisseria meningitidis</i>	Cervicovaginal epithelium	DysA plus	MCP/CD47
<i>Helicobacter pylori</i>	Gastric mucosa	BurA2	Fibronectin
<i>Campylobacter jejuni</i>	Upper airway tract epithelium	PAP	α -D-glucosyl galactosylmannan
<i>Neisseria meningitidis</i> serotype 4	CNS epithelium	S-tropin	ICAM-1
<i>Moraxella catarrhalis</i>	Buccal and bronchial epithelium	Haemagglutinating pilin	The A-antigen of <i>S. pneumoniae</i>
<i>Yersinia sp.</i>	Human B-cells	Invasin	B1 integrins

Table 1.5 Examples of Bacterial Adherence Mechanisms

A selection of bacterial-host relationships in which the molecular basis has been defined

Pathogen	Host Tissue	Adhesin	Receptor	Reference
<i>Streptococcus pyogenes</i>	Extracellular matrix	Protein F	Fibronectin	(Hanski and Caparon, 1992)
<i>Enterobacter cloacae</i>	GIT epithelium	Mannose sensitive pilus	Man, (GlcNAc) ₂ oligosaccharides	(Pan et al., 1997)
<i>Neisseria gonorrhoea</i>	Genito-urinary tract	Type 4 pilus	MCP/CD46	(Kallstrom et al., 1997)
<i>Helicobacter pylori</i>	Gastric mucosa	BabA2	Fucosylated glycoprotein	(Ilver et al., 1998)
Uropathogenic <i>E. coli</i>	Upper urinary tract epithelium	PAP	α -D-galactopyranosyl-(1-4)- β -D-galctopyranononose(Gal α (1,4)G al)	(Stapleton et al., 1998)
Meningitis causing <i>E. coli</i>	CNS epithelium	S fimbriae	sialyl- α -2-3-galactosides	(Hacker et al., 1993)
<i>Haemophilus influenzae</i>	Buccal and bronchial epithelium	Haemagglutinating pili	The Anton antigen	(Gilsdorf et al., 1997)
<i>Yersinia spp.</i>	Human M-cells	Invasin	β 1 integrins	(Isberg and Leong, 1990)

1.7.2 Mechanisms of EPEC and EHEC Adherence

Although the mechanism of *LEE*-mediated intimate attachment has been well described for both EPEC and EHEC (see section 1.6.2.3), the initial non-intimate (>20nm) adherence steps that are assumed to occur are less well understood. Both EPEC and EHEC strains exhibit the phenotype of localised adherence when added to cultured epithelial cell monolayers. Localised adherence manifests as microcolonies of bacteria clustered together on the host cell surface, as distinct from diffuse adherence where individual bacteria are evenly distributed over the host cell surface (Nataro and Kaper, 1998). In EPEC the localised adherence phenotype is mediated by bundle forming pili (BFP) encoded by genes on the EAF (*E. coli* factor for adherence) plasmid (Giron et al., 1991). It is thought that BFP mediate inter-bacterial adherence resulting in the localised adherence. Although EHEC do not possess the EAF plasmid they do possess the localised adherence phenotype and an equivalent factor to BFP has been proposed to be the fimbriae encoded by one of the chromosomally located fimbrial operons of *E. coli* O157:H7 (Torres et al., 2002). Other putative adherence factors that may play a role in *E. coli* O157:H7 colonisation include: IHA (Tarr et al., 2000), *E. coli* factor for adherence (Efa-1) (Nicholls et al., 2000) and other fimbrial operons identified by genome sequences including two with homology to LPF of *Salmonella typhimurium*. Surface organelles morphologically similar to LPF were expressed by *E. coli* O157:H7 when a key regulator for the *LEE*, the *LEE*-encoded regulator (LER) was deleted (Elliott et al., 2000). A homologue of LPF has been described in *LEE* negative EHEC strains (Doughty et al., 2002) and deletion of the version in *E. coli* O113:H21 (a *LEE* negative VTEC) reduced adherence to cultured epithelial cells.

It is possible that *LEE* encoded factors, for example intimin or EspA filaments, may mediate the initial adherence step and therefore different subtypes of these factors could mediate tropism for different host or tissue types. There is some evidence that intimin type mediates tropism for human intestinal explants from different locations. An explanation for intimin mediated tissue tropism is the hypothesis that a separate portion of the molecule from that which binds Tir (the C-type lectin like domain) has an affinity for a host cell receptor. Intimin- γ has been

shown to bind to a molecule expressed on the surface of HEP-2 cells known as nucleolin (Sinclair and O'Brien, 2002).

1.7.3 Fimbrial Adhesins

Type 1 fimbriae of *E. coli* promote adherence to a range of host tissues via mannose sensitive adherence (Ofek et al., 1977). The majority of *E. coli* O157:H7 isolates possess the type 1 fimbrial operon but are incapable of expressing the fimbriae (Enami et al., 1999) due to a conserved deletion in the promoter region preventing off to on switching of the invertible switching element (Roe et al., 2001). The complete genome sequence of *E. coli* O157:H7 has highlighted 14 fimbrial operons (Perna et al., 2001) and two of the fimbrial operons have homology to the long polar fimbriae (LPF) of *Salmonella* spp. (Baumler and Heffron, 1995). An LPF like fimbrial structure has been visualised on an *E. coli* O157:H7 LER mutant by electron microscopy (Elliott et al., 2000). Over-expression of one of the cloned *lpf* operons in an *E. coli* K12 laboratory strain has revealed fimbriae distinct from those described by Elliot *et al* that appear to promote microcolony formation but do not enhance adherence to cultured cell line (Torres et al., 2002). As yet no role for any fimbrial adhesin of *E. coli* O157:H7 in animal or human colonisation has been ascribed.

1.8 Models of Colonisation and Persistence of *E. coli* O157:H7

1.8.1 *In vitro* Systems Used to Examine Colonisation Mechanisms of *E. coli* O157:H7

1.8.1.1 Cell Lines

HEp-2 cells (human laryngeal epidermoid carcinoma) and HeLa (Helen Lane) cells have been used extensively to examine adherence mechanisms of EPEC and EHEC. A/E lesions are readily formed by *LEE* positive strains on these cells and the fluorescent actin stain (FAS) test has been developed to provide a relatively simple assay for the A/E phenotype on such cells (Knutton et al., 1989). Numerous discoveries relating to the mechanism of A/E lesion formation have been made using these cell lines and the role of intimin in the adherence of *E. coli* O157:H7 has been expanded by the observation that a cell surface molecule of HEP-2 cells, nucleolin (Sinclair and O'Brien, 2002), co-localises to intimin. T84 human colonic carcinoma

cells have advantages over the previous cell lines in that they polarize and produce tight junctions on collagen-coated surfaces, producing a monolayer that is physically more like natural epithelium. This however is no guarantee that the expressed cellular receptors reflect those that occur *in vivo*, although the adherence of a range of EHEC strains to these cells correlated to the observed adherence to isolated rabbit colonocytes (Winsor et al., 1992).

1.8.1.2 Primary Epithelial Cells

Cell lines are either naturally occurring tumour cells or cells that have been immortalised *in vitro*. They have the convenience of being sub-culturable indefinitely but may not possess the same compliment of surface receptors as the cells they are supposed to be representing. Alternatively, but less conveniently, primary cell cultures from intestinal epithelium can be used to assess adherence. This has been utilised in one study (Dibb-Fuller et al., 2001) in which *E. coli* O157:H7 formed A/E lesions on bovine primary epithelium with adherence levels higher than EPEC O111, *Salmonella typhimurium* and *E coli* K12.

1.8.1.3 Isolated Viable Intestinal Cells

Intestinal epithelial cells can be separated from underlying connective tissue. Problems with this method include contamination of fibroblasts or other cell types and the fact that basolateral surfaces are exposed in adherence assays. Adherence of a range of EHEC strains to rabbit epithelium included O157:H7 (Ashkenazi et al., 1992). Adherence to cells obtained from young (<1 week) rabbits was lower than to cells from 4 week-old and adult rabbits, suggesting that epithelial receptors utilised by EHEC adherence factors develop with age, which contradicts studies in which neonatal rabbits were more susceptible to EHEC induced pathology.

1.8.1.4 Tissue Explants

Obtaining fresh explants from either recently killed animals or from mucosal biopsies of humans provides epithelial surfaces that have developed *in vivo* and can be maintained *in vitro*. When cultured in appropriate conditions, they can be maintained in a viable state for several hours, a system known as *in vitro* organ

culture (IVOC). IVOC has advantages over conventional tissue culture techniques in that the epithelium is fully differentiated and polarised, all the relevant cell populations such as goblet cells, intra-epithelial lymphocytes and Paneth cells (Porter et al., 2002) are present and an intact normally developed mucus layer is also present. IVOC has been used to examine a range of host epithelium-bacterial interactions, e.g. *Haemophilus influenzae* on respiratory tract epithelium (Middleton et al., 2003), *Pseudomonas aeruginosa* (Esco et al., 2002) on ocular corneal epithelium, *Helicobacter pylori* on gastric mucosa (MeyerRosberg and Berglindh, 1996) and EPEC (Hicks et al., 1998), EAEC (Hicks et al., 1996) and EHEC (Phillips et al., 2000) on intestinal mucosa. A series of studies using human paediatric intestinal mucosa have examined aspects of intimin-mediated attachment *in vitro*. Intimin was shown to be necessary for A/E lesion formation in EPEC O127:H6 (Hicks et al., 1998) and *E. coli* O157:H7 (Fitzhenry et al., 2002). Intimin type dictates which tissue types are susceptible to this adherence mechanism in both EPEC O127:H6 and *E. coli* O157:H7, with native intimin α mediating a greater affinity for small intestinal than large intestinal mucosa and intimin γ mediating a restricted affinity for the Peyer's patch FAE (Fitzhenry et al., 2002; Phillips and Frankel, 2000; Phillips et al., 2000). However the same authors questioned the contribution of intimin type to this tropism in O55 serotypes as four different strains, two expressing intimin γ , two expressing intimin α , all had the restricted tropism for Peyer's patch FAE (Fitzhenry et al., 2002). The experimental output for all these studies was the proportion of explants containing A/E lesions as determined by scanning electron microscopy. Although this is appropriate for determining whether or not the factors involved in A/E lesion formation are functional on a particular tissue type, it may lead to misleading results when studying tissue tropism of the organism itself, especially when this may be affected by non-LEE encoded factors. Quantifying the level of adherence, regardless of the underlying mechanism, would provide a more robust means of determining tissue tropism and the relative contribution of defined factors to this.

Due to its importance as a reservoir for *E. coli* O157:H7, bovine tissue explants have also been used in IVOC to assess bacterial adherence. Phillips *et al*

showed that this organism was capable of forming A/E lesions on bovine Peyer's patch FAE (Phillips et al., 2000) whereas Baehler and Moxley showed A/E lesion formation on both colonic and rectal explants (Baehler and Moxley, 2000). The latter authors have recently developed the culture conditions further and compared different methods of adherence quantification (Baehler and Moxley, 2002). They found A/E lesion formation was optimised by using bacteria cultured in tryptic soy broth, incubation in 5% CO₂ and rocking at 18 cycles per minute. They determined that quantification by "percentage tissue sections positive for A/E lesions" and "number of A/E foci per mm" were the most sensitive methods tested. However these studies included both microcolonies and individual adherent bacteria in their enumeration and assumed they were all intimately attached.

1.8.1.5 Gut Loops

Adding bacteria directly to ligated sections of gut in terminally anaesthetised animals provides an viable epithelial surface *in vivo* system perfused with blood. An advantage of this is that pathological responses can be observed, such as fluid accumulation and influx of polymorphonucleocytes. Gut loops have most successfully been used to study the pathogenesis of *Salmonella spp.* A/E lesions, albeit sparse and small, were demonstrated in 6 month-old sheep colon loops for *E. coli* O157:H7 and *E. coli* O26:H11 after 6 hours (Wales et al., 2002), 30-day old calf ileal loops for *E. coli* O157:H7 and *E. coli* O103:H2 after 12 hours (Stevens et al., 2002) and 2 to 6 day-old calves after 10 hours for O5:H-, O26:H11, O111:H- and O113:H21 (Sandhu and Gyles, 2002).

1.8.2 In Vivo Models of *E. coli* O157:H7 Colonisation

1.8.2.1 Experimental Colonisation of *E. coli* O157:H7 in Cattle

Due to the importance of this species as a reservoir for *E. coli* O157:H7 several groups have attempted to establish bovine models both to reproduce both long-term shedding and intestinal colonisation. Many of the reported calf *E. coli* O157:H7 persistence studies are summarised in table 1.6. Several aspects of the experimental design differ between the various studies such as dose, route of administration, age of animal and the strain used. However the resultant colonisation patterns have certain

common attributes, namely, the lack of any overt clinical disease or pathological changes despite in some instances a single inoculum of 10^{10} CFU, the persistence of the organism in a proportion of individuals for up to several weeks and the lack of detectable A/E lesions on the intestinal epithelium. The duration and magnitude of shedding varies markedly between animals within the same group and day-to-day fluctuation can also be great.

Attempts to localise the colonised bacteria to a region of the GIT have led to conflicting conclusions with one study (Brown et al., 1997) finding the most consistent isolation and greatest numbers in the fore-stomachs and another (Cray and Moon, 1995) reporting a large intestinal location. Taking all the reports collectively, the organism could be recovered from all sites examined, with the exception of the abomasum, presumably because of the inhibitory effect of the low pH. All the post-mortem data in these reports is expressed collectively as a mean or a range, therefore the levels of bacteria within the GIT could not be assessed on an individual animal basis and importantly could not be compared to the levels in the faeces sampled directly before post-mortem. Brown *et al* reported that mucosal washings contained relatively few bacteria and were only positive when the corresponding contents were also positive.

Natural acquisition of *E. coli* O157:H7 has also been reported under controlled conditions (Besser et al., 2001; Shere et al., 2002; Wray et al., 2000) in which the challenge was likely to have been via repeated low doses. This method of challenge can result in a similar magnitude and duration of colonisation as the single large dose used in other studies but is not as reliable. Many individuals are not colonised, which clearly limits the group size and therefore the value of an experiment. A similar problem occurs when small inoculae are used (e.g. 10^4 CFU (Besser et al., 2001)) as again, the proportion of individuals that become colonised is prohibitively low. Regardless of the initial route of administration, even when efforts are made to isolate individuals to prevent re-ingestion of the challenge strain, the possibility of re-circulation of bacteria can never be ruled out. It is possible that re-circulation plays an important role in the persistent colonisation of this organism but this would also occur in the natural on-farm situation and so should not be seen as an experimental flaw. The enhanced persistence of *E. coli* O157: H7 relative to

control strains could be due not only to its ability to colonise and multiply within the host but also to re-circulate, which in turn may be due to an enhanced ability to survive within the external environment or tolerate the acidic gastric conditions during passage through the abomasum.

In an attempt to reproduce in cattle the pathological consequences and extensive mucosal colonisation via A/E lesions reported for neonates of other species, e.g. piglets (Tzipori et al., 1995) and rabbits (Potter et al., 1985), Dean Nystrom *et al* challenged 1 and 3 day old calves with 10^{10} CFU of *E. coli* O157:H7 (Dean-Nystrom et al., 1997). Watery or bloody diarrhoea occurred within 18 hours post-inoculation and histologically, neutrophil infiltration, oedema, villous atrophy and fibrinohaemorrhagic exudates were observed. A/E lesions could be detected at sites in both the small and large intestine. The same group challenged animals of the same age with an intimin deleted *E. coli* O157:H7 and determined that this factor was required not only for A/E lesion formation but also for the pathological and clinical signs of disease observed for the parent strain (Dean-Nystrom et al., 1998). By using such young animals, in which clear pathological changes can be observed and attributed to the challenge bacteria, there is a clear output that can be used to assess the impact of factors such as adhesins or toxins. However these changes are not consequences of experimental or natural infection in older calves and A/E lesions had not been observed in such animals. In order to overcome this the same workers made use of the fact that *E. coli*, including O157:H7 (Harmon et al., 1999), are shed from cattle at much higher levels following a period of fasting. Watery diarrhoea and intestinal damage less severe to that observed in neonates was reproduced in fasted weaned calves and A/E lesions were detected (Dean-Nystrom et al., 1999a). This pathology was later shown to require intimin (Dean-Nystrom et al., 1999b). Both these situations deviate from those encountered naturally in that observations are made soon after challenge with large inoculae in conditions that favour the widespread multiplication of the challenge bacteria. As described above, several studies have examined long-term persistence of the organism in healthy weaned calves and adult cattle using a variety of inoculation methods. Although many of these used large initial inoculae (10^8 to 10^{10} CFU) to reliably achieve colonisation, the duration and magnitude of the shedding after the initial period of intense

colonisation is more likely to represent the natural conditions of the organism and thus lend validity to any observations made. Like disease in Dean-Nystrom's models, intimin was recently also shown to play a role in persistence in such a model by the deletion of *eaeA* (Cornick et al., 2002).

Many groups have made use of experimental models to examine the effects of non-bacterial factors on *E. coli* O157:H7 persistence in cattle. The findings of Diez-Gonzalez et al (1998) and Tkalcic et al (2000) suggest that the magnitude of faecal shedding of *E. coli* O157:H7 in cattle can be reduced by a diet lower in grain and higher in hay. It is proposed that the higher levels of volatile fatty acids induce acid resistance enhancing the survival in the low pH of the gastric stomach. This hypothesis is contradicted by Hovde (Hovde et al., 1999) who found no difference in acid resistance between isolates from grain fed and hay fed cattle and Hancock *et al* (Hancock et al., 1999) who claim that induced acid resistance has not been shown to influence the infectivity of *E. coli* O157:H7. Hovde *et al* (Hovde et al., 1999) also claimed that an abrupt diet change prior to slaughter reduces volatile fatty acid concentration in the colon, reduces dry matter intake and alters rumen microflora which all increase the risk of *Salmonella* infections. The positive effect of fasting on the shedding levels of *E. coli* O157:H7 in experimentally colonised cattle has been demonstrated (Magnuson et al., 2000) (Reid et al., 2002) although the mechanism behind this is unclear. It could be due to changes in the relative populations of other commensals, a reduction in the concentration of volatile fatty acids (products of fermentation known to be inhibitory to *E. coli* O157:H7), an increase in pH or a reduction in the proliferation and turnover of GIT cells (Magnuson et al., 2000).

The effect of probiotic microbial application on *E. coli* O157:H7 shedding in calves has been examined. Zhao *et al* (Zhao et al., 1998) selected 18 of 1200 isolates from bovine faeces that were inhibitory to *E. coli* O157:H7 growth *in vitro* (all but one were Stx negative *E. coli*). This mixture of strains reduced the magnitude and duration of *E. coli* O157:H7 shedding in experimentally challenged calves although this effect was not compared against non-inhibitory control strains and only 6 calves were used. A probiotic mixture of *Streptococcus bovis* and *Lactobacillus* was shown to completely inhibit shedding of *E. coli* O157:H7 in experimentally challenged

calves (Ohya et al., 2000) that coincided with large increases in faecal volatile fatty acid concentrations.

Author (Year)	Age	Sample Size	Method	Findings
(Wray et al., 2002)	1 yr	6	ATCC 49619	High
(Shore et al., 2002)	1 year	6	ATCC 49619	High
(Gomez et al., 2002)	1 year	4	ATCC 49619	High
(Graham et al., 2002)	1 year	16	ATCC 49619	High

High - Not Enumerated
 ND - Not Determined

Table 1.6 Published Models of Persistent Colonisation of *E. coli* O157:H7 in Cattle

Reference	Animals		Challenge			Shedding on Day 14 or Later	
	Age	Number Challenged	Strain	Dose	Method	Magnitude (CFU/g)	No. Colonised @ D14
(Cray and Moon, 1995)	3-14 weeks	8	3081	10 ¹⁰ CFU	Bottle	2.2.x10 ² to 5.7x10 ⁵	8
	1-3 years	9		10 ¹⁰ CFU	Gavage	<5.0x10 ¹ to 2.5x10 ²	9
		5		10 ⁷ CFU	Gavage	<5.0x10 ¹	2
		5		10 ⁴ CFU	Gavage	NA	0
(Brown et al., 1997)	6-8 weeks	9	5 strain mix	10 ¹⁰ CFU	Gavage	5.0x10 ¹ to 3.1x10 ²	6
(Harmon et al., 1999)	8-12 weeks	9	5 strain mix	10 ¹⁰ CFU	Intra-Rumen	Neg to 3.9x10 ³	NR
(Ohya et al., 2000)	3 months	3	MN157	10 ¹⁰ CFU	Gavage	3.0x10 ⁴ to 5.5x10 ⁵	3
(Sanderson et al., 1999)	1 week	4	86-24	5x10 ⁸ CFU	Gavage	Approx 10 ⁵	4
(Wray et al., 2000)	13-30 days	6	A84/92	2x10 ⁸ CFU	Bottle	ND	5
	Adult	6		10 ⁹ CFU	Feed	ND	4
(Shere et al., 2002)	Weaned	8	NR	NR	Natural	6.0x10 ¹ to 6.8x10 ⁵	5
(Cornick et al., 2002)	1 year	8	86-24	10 ¹⁰ CFU	Oral	0.8x10 ¹ to 2.0x10 ³	8
(Grauke et al., 2002)	1 year	18	ATCC 43894/5	NR	Gavage or Intra-Rumen	Neg to 4.5x10 ⁴	12

NR – Not Reported

ND – Not Determined

1.8.2.2 Sheep (*Ovis aries*)

Sheep have been used as experimental models as the organism presumably requires similar colonisation factors as it does in cattle. The sheep model has advantages over the bovine model as animals of the same age are smaller and larger group sizes can be challenged given the same resources. Wales *et al* challenged both 6-day (Wales *et al.*, 2001) and 6-month old sheep (Wales *et al.*, 2001) with *E. coli* O157:H7 and observed high level shedding and the presence of organism on extensive regions of the GIT. A/E lesions were only detected in the 6-day old animals. The persistence of *E. coli* O157:H7 in sheep relative to other *E. coli* pathotypes was examined (Cornick *et al.*, 2000) and showed a tendency to persist for a longer period, even when inoculated at lower levels (10^7 versus 10^{10} CFU), suggesting a specific adaptation to the ruminant GIT.

1.8.2.3 Swine (*Sus scrofa*)

The gnotobiotic piglet model has been used to demonstrate the importance of *eaeA* for A/E lesions and intestinal disease (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Tzipori *et al.*, 1995). Gnotobiotic piglets infected with either *E. coli* O157:H7 or *E. coli* O26:H11 have been used to provide a model for Stx mediated disease, with intestinal pathology and thrombotic microangiopathy observed (Baker *et al.*, 1997; Gunzer *et al.*, 2002). The ability of *E. coli* O157:H7 to persistently colonise 3 month-old pigs up to 2 months post-inoculation was demonstrated (Booher *et al.*, 2002). The porcine model appears to superficially resemble the colonisation observed for weaned calves and the only fundamental objection to using this species as a model of bovine *E. coli* O157:H7 persistence are the physiological differences between the porcine digestive system (an omnivorous monogastric) and the bovine digestive system (a herbivorous ruminant).

1.8.2.4 Rabbit (*Oryctolagus cuniculus*)

Rabbits are susceptible to EPEC infection and the importance of A/E lesion has been demonstrated. Indeed a separate pathotype of *E. coli*, RDEC/REDEC has been defined for those strains that occur naturally. The infant rabbit was the first animal model used to assess the pathogenicity of *E. coli* O157:H7 (Potter *et al.*, 1985).

Colonisation of the GIT coincided with the development of non-bloody diarrhoea contrasting with the lack of disease in naturally colonised cattle. Rabbits have since been used to examine various aspects of VTEC pathogenesis, for example the protective effect of *Lactobacillus casei* (Ogawa et al., 2001) and the effect of antibiotic therapy on disease progression (Johnson and Boedeker, 2000), although no attempt to re-create the persistent asymptomatic carriage observed for cattle has been reported.

1.8.2.5 Mice (*Mus musculus*)

Due to their size and rapid reproductive rate, mice have traditionally been the most extensively utilised animal model to study a wide range of disease. Mice have been used as a model for human EHEC disease with *E. coli* O157:H7 but relatively large inoculae were required for effective colonisation, e.g. 10^{10} cfu. One group (Wadalkowski, 1990) used streptomycin treatment and a period of fasting prior to intra-gastric inoculation to optimise the uptake of the organism and produce clinically apparent disease. The clinical signs included abnormal faeces with neurological and systemic signs thought to be mediated by systemically absorbed Stx. More recent studies using CD-1 mice (Conlan et al., 2001, Conlan and Perry, 1998) have observed transient colonisation of *E. coli* O157:H7 for two and five weeks respectively. One study (Nagano et al, 2003) in which mice were colonised for up to 3 weeks demonstrated the importance of the *LEE* using a *sepL/EspA* knock-out mutant and demonstrating A/E lesions on the caecal mucosa.

Anti-Stx antibodies provided protection from the systemic effects of colonisation both for *E. coli* O157:H7 (Karpman et al., 1997; Sheoran et al., 2003; Mukherjee et al., 2002) and an EHEC strain, O91:H21 (Lindgren et al., 1993). LPS was also shown to be essential for pathogenesis, as disease was not induced in LPS non-responder mice (Karpman et al., 1997). The mouse model has been used to test anti-O157 LPS vaccines (Bennet-Guerro et al., 2000; Conlan et al., 2001).

1.8.2.6 Chicken (*Gallus gallus*)

A convenient model for bacterial colonisation studies is the 1 day-old chick model. Effective colonisation by *E. coli* O157:H7 was achieved by challenge with inoculae as low as 2.6×10^1 CFU for up to 3 months and for up to 11 months with 10^8 CFU (Schoeni and Doyle, 1994). The lack of a pre-existing microflora is probably the reason for successful colonisation in this system with the organism becoming the dominant coliform and colonising as a conventional commensal independently of any interaction with the epithelial surface. This makes this an inappropriate model for identifying factors required for colonisation in cattle, as colonisation is likely to occur anyway. This is supported by the fact that an intimin negative derivative was not impaired in its colonisation of chicks relative to its parent strain Best *et al* (unpublished). Challenge of older (80 day) chicks with a range of STEC failed to result in pathology or A/E lesions at 10 days post challenge (Sandhu and Gyles, 2002). There are likely to be many fundamental differences in the digestive system physiology between birds and mammals.

1.9 Summary of Published Information About *E. coli* O157:H7 as Known at the Commencement of This Study

The role of cattle as the principal reservoir of what is a significant and potentially lethal human pathogen was well established and a variety of transmission routes had been described. Sensitive isolation and detection techniques had been developed and on-farm prevalence, at least within the US and UK, were estimated to be around 10% overall. Stx was the most important virulence factor in humans and the absence of disease attributed to this serotype in cattle lead to the assumption that they were somehow insensitive to Stx, possibly due to the absence of GB₃ receptors. Many other STEC serotypes were known to be asymptotically carried by cattle and the reason for the increased incidence of *E. coli* O157:H7 associated human disease relative to these was not known. *E. coli* O157:H7 presumably possesses additional factors that either enhance its virulence to humans or its survival within cattle.

The ability to form A/E lesions was assumed to be essential at least to colonise the GIT and possibly also contribute to intestinal pathology in the human host. The molecular basis to A/E lesion formation was well described for EPEC and

although certain differences were described, *E. coli* O157:H7 formed A/E lesions in essentially the same way as EPEC. EPEC possess bundle-forming pili that are thought to mediate the localised adherence pattern via inter-bacterial adherence and may account for their extensive intestinal colonisation during infection. The importance of intimin in determining the distribution of EPEC and associated pathology within the GIT of experimentally challenged animals, together with the fact that *E. coli* O157:H7 possesses an almost unique intimin type, raised the question of whether intimin γ was responsible for its success in cattle and its virulence in humans. Intimin was known to be essential both for the production of A/E lesions and the induction of disease in neonatal calves although its contribution to the long term persistence of the organism in older animals was not established and the fact that A/E lesions had never been reported in persistently shedding nor naturally colonised cattle raised doubts as to the importance of this adherence mechanism in the bovine. It remained possible that *E. coli* O157:H7 did not require any adherence step in order to persist and continue to be shed from cattle and that its ability to compete for nutrients and multiply within the lumen contents was sufficient to explain its prevalence within this host.

1. Demonstrate the necessity of adherence to the bovine intestinal epithelium of *E. coli* O157:H7 in establishing and maintaining its persistence within the lumen by either determining the contribution of a known adhesin or by demonstrating the association of the organism with the epithelial surface in persistently shedding animals at post mortem.
2. Examine the distribution of *E. coli* O157:H7 within the gastrointestinal tract of persistently shedding cattle at post mortem to identify a tropism for any region (e.g. rumen or large intestine) or compartment (i.e. digesta, mucus layer or epithelial surface) that may be mediated either by an adherence factor or the ability to preferentially compete for a particular nutrient source.
3. Compare the persistence and virulence of *E. coli* O157:H7 and *E. coli* O157H- (an STXc isolated from a calf with diarrhoea) within the bovine GIT both to fulfil Koch's postulates for the latter and to provide a rationale for further comparative studies of these two strains that could potentially lead to the identification of novel virulence and colonisation factors.

1.10 Aims of Thesis

1. Establish an *in vitro* model to quantify and examine adherence of *E. coli* O157:H7 to bovine intestinal mucosa. This should represent as accurately as possible the epithelial surface encountered by the bacteria during the course of natural colonisation.
2. Make use of the above model to examine the contribution of defined bacterial factors to the adherence of *E. coli* O157:H7 to bovine intestinal epithelium.
3. Make use of the above model to identify any tropism for mucosa in a particular region of the gastrointestinal tract that could indicate the presence of a tissue specific adherence factor.
4. Establish an *in vivo* system that reliably recreates the persistent, asymptomatic shedding of *E. coli* O157:H7 that occurs naturally within the bovine host.
5. Make use of the above system to examine the contribution of defined bacterial factors to the persistence of *E. coli* O157:H7 within the bovine gastrointestinal tract.
6. Demonstrate the necessity of adherence to the bovine intestinal epithelium of *E. coli* O157:H7 in establishing and maintaining its persistence within the bovine host either by determining the contribution of a known adhesin or by demonstrating the association of the organism with the epithelial surface in persistently shedding animals at post mortem.
7. Examine the distribution of *E. coli* O157:H7 within the gastrointestinal tract of persistently shedding cattle at post mortem to identify a tropism for any region (e.g. rumen or large intestine) or compartment (i.e. digesta, mucus layer or epithelial surface) that may be mediated either by an adherence factor or the ability to preferentially compete for a particular nutrient source.
8. Compare the persistence and virulence of *E. coli* O157:H7 and *E. coli* O26:H- (an STEC isolated from a calf with diarrhoea) within the bovine GIT both to fulfil Koch's postulates for the latter and to provide a rationale for further comparative studies of these two strains that could potentially lead to the identification of novel virulence and colonisation factors.

All chemicals are from Sigma-Aldrich unless otherwise stated.

2.1 Bacterial Strains

A description of the strains used is contained in the relevant sections and a summary is provided as a table (table 2.1).

2.2 Plasmid Constructs

All plasmid constructs used in this work are described in table 2.2.

2.3 DNA Manipulation

Unless otherwise stated, all procedures were performed essentially as described by Sambrook et al. (1989).

2.3.1 Polymerase Chain Reaction

PCR was performed in sterile PCR tubes and the following components added per 50 μ l reaction mix in the order listed:

Materials and Methods

10x dNTP mix 5 μ l

Primer A 5' 100 pmol in 0.5 μ l

Primer B 3' 100 pmol in 0.5 μ l

Taq DNA Polymerase 2.5 U

50 μ l aliquots were placed into PCR tubes and 1 μ l template added to each.

Template DNA consisted of either cell lysates or samples of purified plasmid. Cell lysates were prepared by suspending a portion of a fresh colony into 1 ml MQ water and heating at 90°C for 10 minutes. Template was not added in over of the tubes to get an excess of. The primers used are described in table 2.3.

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2.3.1 Polymerase Chain Reaction

PCR was performed in sterile PCR tubes and the following components added per 50 μ l reaction mix in the order listed:

Double Distilled H ₂ O	38 μ l
10x PCR Buffer	5 μ l
10x dNTP mix	5 μ l
Primer A 5'	100 pmol in 0.5 μ l
Primer B 3'	100 pmol in 0.5 μ l
Taq DNA Polymerase	2.5 U

50 μ l aliquots were placed into PCR tubes and 1 μ l template added to each:

Template DNA consisted of either cell lysates or samples of purified plasmid. Cell lysates were prepared by suspending a portion of a fresh colony into 1 ml MQ water and heating at 90°C for 10 minutes. Template was not added to one of the tubes to act as a control. The primers used are described in table 2.3.

The reactions were placed in a pre-heated PCR block (Bio-rad) and cycles consisted of the following stages, although certain varied according to the primers being used:

Initial Heat Treatment	94°C	4 minutes	
Denaturation	94°C	45 seconds	} x30 cycles
Annealing	x°C	45 seconds	
Extension	72°C	90 seconds	
Final Extension	72°C	10 minutes	

The PCR product was then run on an agarose gel to determine its presence, size and purity. If successful and the product was being used for genetic manipulation it was purified using the Qiagen Qiaprep spin DNA purification kit (Qiagen, UK).

2.3.2 DNA Agarose Electrophoresis

Agarose gels were used to separate and visualise DNA fragments of varying sizes. Gels contained 0.7 to 2 % agarose (according to the size of fragment being visualised) and 1 µl of 1 M ethidium bromide. One of three molecular weight DNA markers were used to determine the approximate size of fragments: λ *Hind*III marker, 1000 base pair marker and 100 base pair marker (Invitrogen). All samples contained 1x DNA loading buffer. Gels were placed in Bio-rad electrophoresis tanks. 10 µl samples were loaded into the wells and covered with 1x TBE. A voltage of 100 V was applied until the dye within the loading buffer reached approximately ¾ the way down the gel. Gels were photographed using a Flowgen MultiImage cabinet.

2.3.3 Plasmid Purification

For medium to high copy number plasmids, bacteria were grown overnight in 5 ml LB with appropriate antibiotic at 37°C at 200 rpm, and 1.5 ml of culture was used to purify the plasmid with the Qiagen Qiaprep Spin mini prep kit (Qiagen, UK)

a final volume of 50 μ l sterile dH₂O. For low copy number plasmids, 5 ml overnight cultures were used to inoculate 50 ml fresh LB with antibiotic, and grown at 37°C at 200 rpm to an OD₆₀₀ of 0.9. The culture was then used to purify plasmids using the Qiagen Qiaprep spin Midi prep kit according to the manufacturers instructions for low copy number plasmids. Plasmid preps were reconstituted in sterile dH₂O. It should be noted that Quiagen do not state what the solutions in the kit are. However the kit is based on the alkaline lysis method described by Sambrook et al. (1989).

2.3.4 Restriction Enzyme Digestions

All digests were performed using restriction endonucleases according to the manufacturers instructions (New England Biolabs (NEB), UK). Typically 1 μ l of specific enzyme reaction buffer was added to a 50 μ l volume containing variable proportions of dH₂O and DNA suspension (depending upon the concentration of DNA in the sample), the appropriate NEB restriction enzyme buffer and in some instances, bovine serum albumen (NEB). Specific reagents and conditions for the endonucleases used in this study are summarized in table 2.4. Digests were incubated in a 37°C water bath for the specified duration. DNA was purified from enzymatic reactions using the Qiagen Qiaprep spin DNA purification kit (Qiagen, UK) according to the manufacturers protocol. Purified DNA was reconstituted using dH₂O.

2.3.5 DNA Purification From Agarose Gels

DNA was purified from agarose gels by excising the required band from the gel with a scalpel after visualisation on a UV transilluminator (SLS, UK). The DNA was then extracted using the Qiagen Qiaprep spin Gel purification kit (Qiagen, UK) according to manufacturers instructions. Purified DNA was reconstituted in 30 μ l of dH₂O.

2.3.6 Ligation of DNA Fragments

Shrimp alkaline phosphatase (SAP, Roche) was used to dephosphorylate digested plasmid DNA to prevent sticky ends of linearised plasmids reforming during ligation reactions. To dephosphorylate DNA, 1 μ l of SAP was added to restriction enzyme digest reactions for the final 30 mins at 37°C and cleaned using the DNA purification

procedure. Digested DNA fragments were visualised on a 0.7% agarose gel, and an appropriate DNA ladder (Life Technologies, UK) and amounts of DNA were estimated to give a 3:1 ratio of insert to vector in a 10 μ l ligation reaction containing 1 μ l of T4 DNA ligase and 1 μ l of 10x ligase reaction buffer (NEB, UK). The reaction was incubated at 16°C overnight.

2.4 Transformations

2.4.1 Preparation and Transformation of Chemically Competent Cells

Bacteria were grown in LB at 37°C with shaking at 200 rpm until an OD₆₀₀ of 0.9. Bacteria were harvested (4000 rpm for 10 minutes at 4°C) and suspended in 10 ml of ice cold TFBI by gentle aspiration. The cells were then harvested by centrifugation (4000 rpm for 10 minutes at 4°C) and suspended in 2 ml of ice cold TFBII.

1 μ l of purified plasmid, was transferred into an aliquot of competent cells, mixed by gentle pipetting, and incubated on ice for 30 minutes in a 1.5 ml Eppendorf tube. The mixture was then transferred to a 42°C water bath for 45 seconds, and then placed on ice immediately. 1 ml of LB was added to the mixture and incubated at 37°C with shaking at 80 rpm for 2 hours. 200 μ l of the reaction was then plated out onto LB plates with appropriate antibiotics and incubated overnight. Colonies were patched onto LB agar, incubated overnight, and then plasmid purification performed to confirm transformation.

2.4.2 Preparation and Transformation of Electro-competent Cells

Bacteria were grown in LB at 37°C with shaking at 200 rpm until an OD₆₀₀ of 0.9. Suspension was transferred to a 50 ml Falcon tube and cooled on ice for 5 minutes. Bacteria were harvested (4000 rpm for 10 minutes at 4°C) and suspended in 25 ml of ice cold 10% glycerol. Bacteria were harvested as above, suspended in 12.5 ml of ice cold 10% glycerol then harvested again and suspended in 2 ml of 10% glycerol.

To a 45 μ l aliquot of cells, 1 μ l of purified plasmid was added, mixed by gentle agitation and incubated on ice for 5 minutes. The mixture was transferred to a 1.5 ml electroporation cuvette (Flowgen, UK) and electroporated at 2.5 kV. One millilitre of LB was added immediately and the suspension transferred to a 1.5 ml Eppendorf tube. The reaction was incubated at 37°C with shaking at 200 rpm for 2

hours and 400 μ l plated on LB agar with appropriate antibiotics. Plates were incubated overnight and transformation confirmed via plasmid purification.

2.5 Methods for Protein Analysis

2.5.1 Whole Cell Lysis

A 1 ml sample of mid-log phase bacteria grown in MEM-HEPES was harvested by centrifugation (4000 rpm for 10 minutes at 4°C) and washed in 0.5 ml 50 mM Tris (pH 7.4). The pellet was resuspended in 25 μ l of water then 25 μ l of 2x SDS gel-loading buffer added. The sample was placed in a boiling water bath for 5 minutes.

2.5.2 Outer Membrane Protein Preparations (Hancock and Poxton, 1988)

Bacterial cultures were grown as above, harvested, washed twice in PBS and resuspended in PBS containing 0.1 mM PMSF (phenylmethanesulphonyl fluoride). The suspension was sonicated (3x 30 seconds at an amplitude of 10 μ m). Unlysed cells were removed by centrifugation (5000 g for 5 minutes) then sarkosyl was added (final concentration of 0.7%) and incubated for 30 minutes at room temperature. After centrifugation (40000 g for 45 minutes) the pellet was resuspended in Tris pH 6.8.

2.5.3 TCA Precipitation of Secreted Protein (McNally et al., 2001)

Bacteria were grown in 50 ml MEM-HEPES to an OD₆₀₀ 0.9. The culture was harvested by centrifugation at 4,000 rpm for 15 minutes at 4°C (Heraus Instruments chilled centrifuge). Culture supernatant was removed and filtered through a 0.2 μ m pore size filter, and 100% TCA was added to achieve a final concentration of 10%. The supernatant was then incubated at 4°C overnight and precipitated protein harvested by centrifugation at 4000 rpm for 30 minutes at 4°C. The supernatant was poured off, the protein pellet air dried for approximately 30 minutes, and reconstituted in 100 μ l of 1.5 M Tris-HCl pH 8.8. The sample was stored at -20°C.

2.5.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples of proteins were analysed by SDS-PAGE and obtained either by whole cell lysis, secreted protein precipitation or membrane preparation. The samples were mixed in an equal volume (usually 8 μ l) with SDS gel loading buffer and heated in a boiling water bath for 10 minutes to denature the proteins and disassociate polypeptides. SDS-PAGE resolving gel was prepared at an appropriate percentage for the size of protein being analysed. The resolving gel was applied to a vertical electrophoresis apparatus (BIO-RAD Mini-protean II gel apparatus, Bio-rad, UK) and once polymerised SDS-PAGE stacking gel was applied to form an upper layer of higher porosity. The apparatus was transferred to an electrophoresis tank (Bio-rad, UK) and tris-glycine-SDS electrophoresis buffer added to the inner and outer compartments. 16 μ l of the sample/loading buffer mixes and 5 μ l of the molecular weight marker (rainbow marker, Amersham Pharmacia Biotech) were added to the wells. A voltage of 100 V was applied until the samples were deposited in a fine boundary on the upper edge of the resolving gel. The voltage was then increased to 150 V until the dye in the samples reached the bottom of the gel.

2.5.5 Colloidal Blue Stain of SDS-PAGE Gels

Gels were removed from the apparatus and transferred to a plastic tray. Colloidal blue (Invitrogen UK) was used to stain gels according to the manufacturers instructions. The gels were stained for 4 hours, and then destained overnight in dH₂O. Gels were photographed using a Flowgen MultiImage cabinet.

2.5.6 Western Blotting

Proteins separated on polyacrylamide gels were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) as follows. The gel, membrane, 4 pieces of 3MM paper (Whatman) and 2 sponge pads were presoaked in transfer buffer and held together in a plastic clamp (Bio-Rad). This apparatus was transferred to a Western blotting tank (Bio-Rad) containing transfer buffer, which was in turn placed on ice. The arrangement of the above apparatus relative to the electrodes was as follows: cathode, sponge pad, 2x 3MM paper, gel, membrane, 2x 3MM paper,

sponge pad and anode. A voltage of 60 V was applied for 90 minutes, after which the membrane was removed and placed in Western blot blocking solution overnight at 4°C.

Dried milk suspension (5%) was washed out with three 5 minute washes in Western blot washing solution. Antibody directed against the target protein was diluted in Western Blot antibody diluent at a dilution that varied according to the antibody being used. The blot was then incubated for 3 hours with the primary antibody at room temperature on a rocking platform. After three 5 minute washes in washing solution the blot was incubated for one hour in HRP (horseradish peroxidase) conjugated secondary antibody, typically swine anti-rabbit IgG (Dako) at a dilution of 1 in 1000 in Western Blot antibody diluent. After three 5 minute washes in washing solution the blot was developed in Luminol Enhancer Solution (Pierce) for 5 minutes, washed briefly in washing solution and placed inside a radiographic cartridge. Radiographic film (Hyperfilm ECL (Amersham Pharmacia Biotech) was laid on top of the blot for variable lengths of time until bands of appropriate strength were produced. The radiograph was developed in a Protec automatic film processor (Optimax).

2.6 Typing Methods

2.6.1 Reverse Passive Latex Agglutination

A test kit (Oxoid) containing anti-Stx antibody coated latex beads was used to detect secreted Stx 1 and Stx 2 to confirm that strains negative by PCR for Stx were in fact negative.

2.6.2 Direct Immunolabelling

To determine the expression of intimin and EspA filaments when experimental strains were grown to mid-log in MEM-HEPES they were labelled directly using antibodies obtained from M. Woodward (CVLA). Cultures were fixed by mixing 1:10 in 4% paraformaldehyde. 20 µl of fixed bacterial suspensions were air dried on glass slides and incubated with the primary antibody (1/10 EspA antibody and neat for intimin antibody) diluted in PBS/0.1% BSA for 60 min at room temperature. After three washes with PBS/0.1% BSA, the samples were incubated with FITC-

conjugated goat anti-rabbit immunoglobulin (1/500, Sigma) for 30 min and the slide washed three times with PBS/0.1% BSA. The slide was then examined by fluorescence microscopy using appropriate filter sets and the images captured using Leica Qfluor software.

2.6.3 Phage Typing (Frost et al., 1993)

Phage typing was performed by Lesley Allison at the Scottish *E. coli* O157:H7 reference laboratory, Edinburgh.

2.6.4 Polymerase Chain Reaction

Published primer pairs and conditions were used to determine the presence of certain genes during the characterisation of the strains used (table 2.3). The PCR reaction and agarose gel electrophoresis methods are described in sections 2.3.1 and 2.3.2.

2.6.5 Stx typing – The Lin system (Bastian et al., 1998)

Stx primers (table 2.3) were used to amplify an 895 base pair region of the Stx genes. The products were digested with HincII (table 2.4) to produce a Stx type specific restriction fragments. Stx 1 gave 705, 158 and 32 bp bands, Stx 2 gave 555, 262 and 78 bp bands and Stx 2c gave 555, 324 and 16 bp bands.

2.6.6 Pulsed Field Gel Electrophoresis (Gautom, 1997)

Strains to be tested were incubated overnight in 5 ml LB broth at 37°C without aeration. Cultures were centrifuged (3400 g for 10 minutes) and the pellet re-suspended in 4 ml SE. The suspension was centrifuged again (3400 g for 10 minutes) then re-suspended in 2 ml SE to give an OD₆₀₀ of approximately 1.4 and incubated for 10 minutes at 70°C. Molten 1.5% chromosomal grade agarose (Bio-Rad Laboratories), in 0.5 ml TE, was mixed with 0.5ml of each cell suspension. Each agarose/strain mix was added to gel plug moulds (Bio-Rad Laboratories) and left to set at room temperature. Plugs were transferred to lysis buffer containing proteinase K (1 mg/ml) and incubated overnight at 55°C. The plugs were then washed for 8x 30 minutes in TE and 2x 15 minutes in 500 µl *xbaI* buffer. 100 µl *xbaI* reaction mix (500 units/ml *xbaI* (New England BioLabs) in *xbaI* reaction

buffer) is added to each plug and incubated overnight at 37°C. Plugs were washed in molecute water 4x 15 minutes then 30 minutes in 0.5x TBE. The electrophoresis gel was prepared using 1% pulsed field certified agarose (Bio-Rad Laboratories) in 100 ml 0.5x TBE. A CHEF mapper system (Bio-Rad Laboratories) PFGE tank was filled with 2 litres 0.5x TBE and run until the temperature reached 14°C. Each plug was transferred to a well in the PFGE gel with a Lamda Ladder PFGE molecular weight marker (N0340S, New England Biolabs) in the wells at either side of the samples. Wells were topped up with molten agarose and the gel was transferred to the tank.

The electrophoresis was run with the following settings:

Initial switch time	2.16 s
Final switch time	54.17 s
Run time	22 h
Angle	120°
Gradient	6.0 V/cm
Temperature	14°C
Ramping factor	linear

The gel was stained with 300 ml 0.5 µg/ml ethidium bromide for 30 minutes followed by a 30 minute washing in distilled water to destain if necessary. Gels were photographed using a Flowgen MultiImage cabinet.

2.6.7 Fluorescent Actin Staining (FAS) (Knutton et al., 1989)

Cells were infected as described, and at the desired time point washed 3 times with PBS and fixed with 4% PFA for 20 minutes. The cells were permeabilised with 0.1% Triton -x-100 in PBS for 5 minutes and incubated in 5 µg/ml FITC-phalloidin in PBS for 20 minutes. Cells were observed using a Leica DMBL fluorescent microscope.

2.7 IVOC Methods

2.7.1 Bacterial Strains

Five wild type *E. coli* O157:H7 strains were selected. These differed markedly in their ability to secrete *LEE* encoded proteins (McNally et al., 2001). ZAP 41 and 46 are bovine isolates that carry the *LEE* and the plasmid pO157, are able to form A/E lesions on HeLa cells but are low *LEE* protein secretors. ZAP 3 is a bovine isolate which was presumed the cause of human outbreak cases identified by trace back and pulsed-field gel electrophoresis of isolates to Lothian Red House dairy (Allison et al., 1998). ZAP 58 is another cattle isolate that was similarly identified as the cause of a human outbreak by the Scottish *E. coli* reference laboratory. ZAP 3, 58 and 26 (*E. coli* O157:H7 EDL933) are all high *LEE* protein secretors and possess pO157. ZAP 27 was used as a negative control for the contribution of *LEE*. The strain is a *LEE* negative mutant of ZAP 26, constructed using double-stranded break allelic exchange (Posfai et al., 1999; Posfai et al., 1997). All strains were grown for inoculation of mucosal explants as follows: a fresh colony was picked from horse blood agar (Oxoid) for inoculation of 5 ml of MEM-HEPES. After overnight incubation (37°C, 200 rpm) 1 ml was used to inoculate 4 ml of pre-warmed MEM-HEPES and incubated for 3 hours (37°C, 200 rpm.). All the strains reached an OD₆₀₀ (optical density at 600 nm) of approximately 0.5 immediately prior to application to explants of the tissue samples.

2.7.2 Statistical Analysis

The study was designed to examine the interaction of 6 different bacterial strains at 4 separate sites from different animals where variability was likely to occur both between and within the individual animals from which the tissues were derived. Logistical constraints did not allow the examination of all combinations of strain and site from each individual animal. A balanced factorial in incomplete block design was therefore designed for the experiment (Mead, 1990). Site and strain interactions were examined by applying the 6 strains to 12 random samples collected from the four sites from each of 6 animals. The strains' potential tropism was quantified by examining a number of microscope fields for each of 10 sections per sample, and determining whether each field contained epithelial associated bacteria. This method

was chosen in preference to the counting of individual bacteria as the enumeration of bacterial numbers in clusters or micro-colonies was considered likely to result in inaccuracy or bias. The use of electron microscopy was precluded to take advantage of the larger areas that can be examined by light microscopy. The data were analysed using a generalized linear mixed model (Brown and Prescott, 1999) in Genstat, fitting a complementary log-log link function with a Bernoulli error distribution.

2.7.3 Tissue Explants

Six Holstein/Friesian male calves were conventionally reared. They were all within the age range of 10-14 weeks and had been weaned at 6 weeks of age. Faeces collected post-mortem were screened for *E. coli* O157:H7 using immunomagnetic separation and were all negative. The calves were euthanased on farm using 20 ml intravenous Somulose (8 g quinalbarbitone, Arnolds Veterinary Products) and the tissue excised and put into ice-cold IVOC culture medium with minimal delay. Full thickness pieces of intestine were taken from the spiral colon, the pre-pelvic rectum and both the Peyer's patch and normal absorptive epithelium of the terminal ileum. 4x4 mm pieces of intestinal mucosa were separated from the underlying muscle with scalpel blades and placed on individual sterile foam pads for subsequent *in vitro* culture. The time between death and setting up the cultures was always less than 1 hour.

2.7.4 *In vitro* Organ Culture

Pre-warmed IVOC culture medium (45% v/v DMEM (Dulbecco's modified eagles medium, 45% v/v NCTC-135 medium 10% v/v newborn calf serum was added until the fluid surface was in contact with the tissue edge. 50 μ l of bacterial culture in mid-logarithmic growth phase was added to each explant and the samples incubated at 37°C in an atmosphere of 95% v/v O₂, 5% v/v CO₂ for 8 hours. Culture medium was changed every 2 to 3 hours. At the end of the culture period the samples were washed once in fresh culture medium and fixed in 4% w/v paraformaldehyde in PBS.

2.7.5 Quantification of Bacterial Mucosal Affinity

The tissue samples were embedded in paraffin blocks and routine histological sections cut. Bacteria on the sections were labelled by indirect immunofluorescence using rabbit anti-O157 antisera (1 in 125) (Mast Diagnostics) and FITC-conjugated goat anti-rabbit secondary antibody (1 in 1000). Anti-O26 antisera from the same supplier failed to label bacteria on samples known to contain *E. coli* O157. Tissue was visualized by counterstaining with propidium iodide ($1 \mu\text{g ml}^{-1}$) and viewed on a Leica DMLB epifluorescent microscope using a 40x objective. Observation was conducted without knowledge of the bacterial strain present on the samples.

2.7.6 Electron Microscopy

Bacterial cultures were prepared as described above and fixed by the addition of an equal volume of 3% w/v glutaraldehyde. The fixed suspension was immobilized onto carbon formvar-coated copper slots (Agar Scientific) and negatively stained with 1% w/v phosphotungstic acid (pH 4.0). Selected tissue blocks were deparaffinised with xylene, re-hydrated in graded dilutions of ethanol and post-fixed in osmium tetroxide. After dehydration in graded dilutions of acetone the samples were infiltrated and embedded in araldite. Ultra-thin sections (80 nm) were cut and mounted on copper grids (Agar Scientific). All specimens were viewed and photographed on a Philips CM12 transmission electron microscope.

2.8 Methods for Calf Colonisation

2.8.1 EHEC Strains

E. coli O157:H7 strains used for oral inoculation of calves were ZAP 196, ZAP 198 and ZAP 3. ZAP 196 and ZAP 198 were both isolated from the same human patient in Washington State (USA). Cattle were established as the source of this outbreak. ZAP 196 possesses Stx2 while ZAP 198 was naturally cured of the Stx2 bacteriophage. They are otherwise indistinguishable as determined by phage typing (both type 32), LEE protein secretion level and pulsed-field gel electrophoresis (both untypable). ZAP 3 (Scottish *E. coli* O157:H7 reference laboratory number – 659) is a bovine isolate from Red House Dairy (Blackburn, West Lothian, Scotland) that

caused a milk-borne human outbreak. The strain used for challenge via co-habitation with a shedding calf at Washington State University was WSU 2043. This was originally isolated from an asymptomatic calf and possesses the genes for Stx1, Stx2 and Stx2c. All these strains were selected for spontaneous resistance to nalidixic acid to facilitate recovery from GIT contents and tissues. The strain isolated from a naturally colonized 12 month old steer on a farm in Inverness-shire has been designated ZAP 278. It possesses genes for Stx2 and Stx2c and is phage type 21/28. All strains used have been shown by PCR using published primers to possess genes for enterohaemolysin, intimin- γ , EspA and EspB. *E. coli* O26:H- (Ebel et al., 1996; Wieler et al., 1996) was originally isolated from a diarrhoeic calf and its *LEE* is currently being sequenced. In addition, it possesses Stx1, intimin β , a plasmid encoded enterohaemolysin and type 1 fimbriae.

2.8.2 Generation of Nal^R Derivatives

In order to isolate challenge strains from faecal samples and to permit enumeration on direct culture a selective medium is required. The use of cefixime-tellurite is extensively used to select for *E. coli* O157:H7 from field samples but contaminants are frequently cultured which confound enumeration. By selecting spontaneous mutants that are resistant to particular anti-microbial agents the challenge strains can be marked to facilitate recovery and enumeration. This also prevents confusion with any wild-type *E. coli* O157:H7 present, although these should not occur as only *E. coli* O157:H7 free animals are used for challenge. Nalidixic acid (a fluorquinolone) is frequently used to mark challenge strains since natural resistance is rare and it is relatively cheap and easy to prepare. ZAP 193 was obtained as a Nal^R derivative of NCTC 12900. All the other strains used for calf challenges were derivatives of Nal^S strains selected for spontaneous resistance to nalidixic acid as follows: A sweep of colonies were used to inoculate 10 ml Luria Bertani broth and cultured overnight at 37°C shaking at 200 rpm. The culture was centrifuged (4000 g for 5 minutes), the supernatant was removed and the bacterial pellet suspended in 1ml sterile PBS. 200 μ l of the bacterial suspension was spread onto each of 5 LB agar plates containing 15 μ g/ml nalidixic acid. The plates are incubated overnight at 37°C and any colonies present are spontaneous mutants resistant to nalidixic acid. Some of these colonies

are selected for storage at -70°C in Protect Vials and subcultured in LB broth containing 15µg/ml nalidixic acid as described above. The overnight growth confirms the strain's resistance to nalidixic acid.

2.8.3 Calf colonization

Separate experimental calf challenges used either ZAP 3, ZAP 196 or ZAP 198 and were performed at Moredun Research Institute (MRI) in either containment level 2 or 3 large animal housing facilities under Home Office license number 60/02105. Calves were reared conventionally on a farm until at least 2 weeks post-weaning and transported to MRI where they were acclimatized for at least three days prior to challenge. Prior to challenge faecal samples were taken at least twice from each calf and confirmed negative for *E. coli* O157:H7 by immuno-magnetic separation (IMS). At the time of challenge the calf ages ranged from 8-14 weeks.

The challenge *E. coli* O157:H7 strain, was grown overnight in Luria Bertani (LB) broth (37°C, with aeration) and diluted in sterile PBS to achieve an inoculum of 10⁹ CFU per animal in a total volume of 10 ml. The inoculum was administered to the calves via stomach tube and washed down with 500 ml sterile PBS. Faeces were caught or collected per rectum for culture and bacterial enumeration.

Experimental calf challenges with WSU 2043 were performed at Washington State University by T.E. Besser *et al* using a direct contact infection model system (Besser *et al.*, 2001). Briefly, groups of calves were first confirmed to be free of detectable *E. coli* O157:H7 shedding as described above. Then, a single calf was removed from the group and experimentally challenged by an inoculum prepared as described for the above calves but administered orally by syringe. The re-introduction of the inoculated calf into the group pen constituted the challenge and the subject calves were monitored for acquisition of *E. coli* O157:H7 infection.

2.8.4 Microbiology

10 g quantities of faeces or GIT contents were suspended in 90 ml sterile PBS and serially diluted in 10-fold steps in PBS. These serial dilutions were cultured as 100 µl aliquots spread in duplicate or triplicate onto sorbitol MacConkey agar (Oxoid) plates containing 15 µg ml⁻¹ nalidixic acid (N-SMAC). All inoculated media were

incubated overnight at 37°C. Non-sorbitol fermenting colonies on N-SMAC plates were counted and 1-3 colonies from each sample tested for O157 LPS antigen using a latex agglutination test kit (Oxoid). The most probable number of colony forming units was determined as described under statistical methods. For enrichment cultures 1 ml of the neat suspension or tissue washing supernatant were added to 9 ml of LB containing 15 µg ml⁻¹ nalidixic acid. The enrichment cultures for samples negative on direct plating were spread onto fresh N-SMAC plates and incubated overnight at 37°C. These procedures are reported to be at least as sensitive as IMS for detecting marked strains (Sanderson et al., 1995).

2.8.5 Statistical Methods

The most probable numbers of colony forming units were determined by fitting generalized linear models with a Poisson error distribution and logarithmic link function while incorporating the logarithm of dilution as an offset variable (McCullagh and Nelder, 1989).

2.9 Localisation of *E. coli* O157:H7 in Shedding Cattle

2.9.1 Animals

Experimentally challenged individuals consistently shedding the organism prior to the chosen date of necropsy were selected for sampling to determine the location of *E. coli* O157:H7 within the GIT. This was typically 21 to 28 days post challenge but one individual was sampled at day 14. The 12-month-old steer that was naturally shedding >10⁴ CFU/g of *E. coli* O157:H7 was identified on a farm in Inverness-shire by field epidemiology work performed by M. Pearce (SAC, Inverness).

2.9.2 Necropsy Procedure

The precise procedure varied, with more detailed analysis of the terminal rectum being performed as the tropism of *E. coli* O157:H7 became better defined with each necropsy. The most complete necropsy procedure is described here with an individual description of each necropsy included in the results section. As close as possible to killing, a sample of naturally passed faeces was caught with a fresh glove, and split into surface and core components by dissection with a sterile scalpel. This

procedure was not performed for animals sampled prior to calf 299. In addition, certain subsequent ante-mortem faeces were too fluid to be split into their surface and core components. In these cases faeces containing an undetermined mixture of surface and core were used for enumeration and described as "whole faeces". Following euthanasia with intravenous pentobarbitone (supplier) the abdomen was opened. The terminal 20 to 30 cm of rectum and anus were removed as a single piece after the rectum was double ligated and transected, the anus circumsected and the pubic bone reflected. This length of gut was opened longitudinally in a proximal to distal direction. The recto-anal junction (RAJ) was used as an anatomical marker from which measurements were made to define anatomical locations. It is grossly visible being the interface between colorectal columnar epithelium and anal stratified squamous epithelium. 10 g of the distal-most contents were taken and their locations relative to the RAJ noted, although these varied from animal to animal. When present 10 g samples of lumen contents were taken from between 30 cm and 20 cm proximal to the RAJ. Samples of tissue visibly free of faeces were taken from the following portions of rectum relative to the RAJ (in cm): 20-30, 10-20, 5-10, 3-5, 1-3 and -1+1. Lumen contents and tissue were also taken from the following locations: rumen (cranio-ventral sac), ileum (10 cm proximal to the ileo-caeco-colic junction, including both normal absorptive mucosa and Peyer's patch), caecum (apex) and colon (point of inflection of spiral colon). All lumen contents and tissue were processed for microbiological analysis (described below). A sample of each tissue was fixed in 4% paraformaldehyde for cutting of sections to be used for immunofluorescent detection of *E. coli* O157.

2.8.6 Statistical Analysis

2.9.3 Microbiology

Enumeration of *E. coli* O157:H7 from lumen contents was performed as described for faecal enumeration (2.8.4). In addition bacteria washed from mucosal samples was enumerated by vortexing 1 cm² pieces in 5 ml PBS for 60 seconds. 100 µl of neat and 10 fold serial dilutions were plated onto SMAC agar plates and 1 ml inoculated into 9 ml LB broth, as described for the lumen contents/faecal suspensions. Samples for enumeration from the naturally colonized animal were cultured on sorbitol MacConkey agar using cefixime-tellurite (0.05 and 25 µg ml⁻¹

respectively, Oxoid) instead of nalidixic acid selection. Similarly, enrichment was performed with LB containing cefixime-tellurite (the same concentration as above) in place of nalidixic acid. Some samples were also spread on tryptone bile x-glucuronide media (TBx) (Oxoid) to enumerate glucuronidase positive, non-O157:H7 *E. coli* strains (Thompson et al., 1990).

2.9.4 Immunofluorescence Microscopy

E. coli O157 was visualized by fluorescence microscopy of paraformaldehyde-fixed sections after incubation with rabbit anti-O157 polyclonal antibody (Mast-Assure) (1 in 100 for 30 minutes at room temperature), followed by FITC-conjugated goat anti-rabbit antibody (1 in 1000 for 30 minutes at room temperature). Both antibodies were diluted in PBS containing 0.1% bovine serum albumin. Stained sections were viewed on a Leica DMLB epifluorescent microscope with a 40x objective. As a negative control certain samples were also stained with normal rabbit serum or anti-O26 polyclonal antibody (Mast-Assure). For confocal microscopy, tissue was counterstained with TRITC-phalloidin and viewed with a Zeiss 510 confocal microscope with a 63x objective lens.

2.9.5 Electron Microscopy

Tissue blocks on which microcolonies were located by immunofluorescence microscopy were processed for transmission electron microscopy as described in section 2.7.6.

2.9.6 Statistical Analysis

The most probable numbers of colony forming units were determined by fitting generalized linear models with a Poisson error distribution and logarithmic link function while incorporating the logarithm of dilution as an offset variable (McCullagh and Nelder, 1989). Bacterial counts at different sites were compared by fitting a generalized linear mixed model (Brown and Prescott, 1999), with the same error and link functions, while fitting Animal and Animal Site as random effects. The unbalanced nature of the data did not always allow this model to converge to stable values, in which case Animal was fitted as the sole random effect. These

models typically had a moderate to large overdispersion estimate, and these values were always used to estimate the standard errors of the model estimates.

Results from animals sampled by T.E. Besser *et al* at Washington State University were included in the analysis. Of the 15 animals studied, detailed bacterial counts of the form required for statistical analysis were not available for 4 animals (a random drop-out from the sample). A further two animals were identified as exhibiting a colonization pattern qualitatively different to that seen in the others. Only samples from the 9 animals where *E. coli* O157:H7 counts were still increasing between the rectal contents and faeces were used in the statistical analysis of the tissue-associated bacteria.

2.10 Solutions

All chemicals are from Sigma-Aldrich unless otherwise stated.

2.10.1 Phosphate Buffered Saline (PBS) (Oxoid)

137 mM NaCl

10 mM Na₂HPO₄

2.7 mM KCl

1.4 mM K₂HPO₄

Adjusted to pH 7.4

2.10.2 TBE

89 mM Tris (hydroxymethyl) aminomethane (Fisher)

89 mM Boric acid

2 mM Ethylenediaminetetraacetic acid (EDTA)

2.10.3 10x Deoxynucleotide Triphosphate Mix

0.4 μM of dATP, dCTP, dTTP, and dGTP (Roche) in sterile double distilled H₂O.

2.10.4 TFBI buffer

30 mM KC₂H₃O₂

10 mM CaCl₂·2H₂O

100 mM KCl

15% Glycerol

45 mM MnCl₂

2.10.5 TFBII buffer

75 mM CaCl₂

10 mM KCl

15% Glycerol

10 mM 3-(N-morpholino) propanesulphonic acid (MOPS)

2.10.6 SDS-PAGE Resolving Gel

x% Acrylamide / N,N'-methylenebisacrylamide (bisacrylamide) (37.5:1)

0.375 M Tris HCl (pH 8.8)

10% Sodium Dodecyl Sulphate (SDS)

10% Ammonium Persulphate (APS)

0.08% N,N,N',N'-Tetramethylethylenediamine (TEMED)

2.10.7 SDS-PAGE Stacking Gel

5% Acrylamide / Bisacrylamide (37.5:1)

0.125 M Tris HCl (pH 6.8)

10% SDS

10% APS

0.1% TEMED

2.10.8 Tris-Glycine-SDS Electrophoresis Buffer

25 mM Tris HCl (pH 8.3)

250 mM Glycine

0.1% SDS

2.10.9 2x SDS Gel-Loading Buffer

100 mM Tris HCl (pH 6.8)

200 mM Dithiothreitol

4% SDS

0.2% Bromophenol Blue

20% Glycerol

2.10.10 Western Blot Transfer Buffer

39 mM Glycine

48 mM Tris

0.037 SDS

20% Methanol

2.10.11 Western Blot Blocking Solution

5% non-fat dried milk (Marvel)

2.10.12 Western Blot Washing Solution

PBS

0.05% Tween[®] 20 (Polyoxyethylenesorbitan Monolaurate)

2.10.13 Western Blot Antibody Diluent

PBS

0.05% Tween[®] 20

0.1% Bovine Serum Albumin

2.10.14 PFGE SE Buffer

75 mM NaCl

25 mM EDTA

Adjusted to pH 8.0

2.10.15 PFGE TE Buffer

10 mM Tris

0.1mM EDTA

2.10.16 PFGE Lysis Buffer

50 mM Tris

50 mM EDTA

1% Sarcosine

2.10.17 PFGE Wash Buffer

10 mM Tris

1 mM EDTA

Table 2.1 Bacterial Strains Used in This Study

Strain	Original Designation (and Source)	Description (and Reference)
ZAP 1	319 (Scottish Ref. Lab ¹)	<i>E. coli</i> O157:H7 Redhouse dairy outbreak strain: human isolate (Allison et al. 1998)
ZAP 3	659 (Scottish Ref. Lab ¹)	<i>E. coli</i> O157:H7 Redhouse dairy outbreak strain: bovine isolate (Allison et al. 1998)
ZAP 21	413/89-1 (Trinad Chakraborty ²)	<i>E. coli</i> O26:H- Stx1 isolated from a calf with diarrhoea (Djafari et al., 1997)
ZAP 25	(Trinad Chakraborty ²)	<i>E. coli</i> 413/89-1 Δ <i>eaeA</i>
ZAP 26	EDL933 (Gyorgy Posfai ³)	<i>E. coli</i> O157:H7 linked to outbreak of HC (Perna et al., 2001)
ZAP 27	(Gyorgy Posfai ³)	<i>E. coli</i> EDL933 Δ <i>LEE</i> Cm ^r (Posfai et al., 1997)
ZAP 41	6303 (Scottish Ref. Lab ¹)	<i>E. coli</i> O157:H7 bovine isolate
ZAP 46	6043 (Scottish Ref. Lab ¹)	<i>E. coli</i> O157:H7 bovine isolate
ZAP 58	6409 (Scottish Ref. Lab ¹)	<i>E. coli</i> O157:H7 Northern Ireland bovine isolate linked to human infection
ZAP 193	NCTC 12900 (Ian Ogden ⁴)	<i>E. coli</i> O157:H7 Stx ⁻ (Dibb-Fuller et al., 2001)
ZAP 195	(Martin Woodward ⁵)	<i>E. coli</i> O80:NM (Pearson et al., 1989)
ZAP 196	Walla 1 (Mary Reynolds ⁶)	<i>E. coli</i> O157:H7 (Ostroff et al., 1990)
ZAP 198	Walla 3 (Mary Reynolds ⁶)	<i>E. coli</i> O157:H7 (Ostroff et al., 1990)
ZAP 201	85-170 (Gad Frankel ⁷)	<i>E. coli</i> O157:H7 Stx- (Fitzhenry et al., 2002)
ZAP 202	ICC 170 (Gad Frankel ⁷)	85-170 Δ <i>eaeA</i>

ZAP 203	ICC 170 (Gad Frankel')	ICC 170 :: pCVD438
ZAP 204	ICC 170 (Gad Frankel')	ICC 170 :: pICC55
ZAP 210	This study	ICC 170 :: pSWN01
ZAP 211	This study	<i>E. coli</i> 413/89-1 Δ eeaeA pSWN01
ZAP 212	This study	<i>E. coli</i> 413/89-1 Δ eeaeA pICC55
ZAP 278	This study	<i>E. coli</i> O157:H7 Stx2/2C isolated from a 12 month old steer in Inverness-shir

1. Scottish *E. coli* O157:H7 Reference Laboratory, Dept Clinical Microbiology, Crewe Road, Edinburgh EH4 2xU
2. Trinad Chakraborty, Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität, D-35392 Giessen, Germany
3. Gyorgy Posfai, Biology Research Centre, Institute of Biophysics, H-6701 Szeged, Hungary
4. Ian Ogdén, University of Aberdeen, Dept Microbiology, Aberdeen AB9 1Fx, Scotland
5. Martin Woodward, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB
6. Mary Reynolds, Centre for Disease Control & Prevent, Atlanta, Georgia 30333 USA
7. Gad Frankel, Dept Biochemistry, Imperial College of Science, Medicine and Technology, London SW7 2AZ

Table 2.2 Plasmids Used in This Study

pACYC184	<i>E. coli</i> plasmid cloning vector, low copy number. Cm ^r , Tc ^r (Sambrook et al., 1989)
pCVD438	pACYC184 encoding intimin alpha from <i>E. coli</i> O157:H7 (Donnenberg and Kaper, 1991)
pICC55	pCVD438 derivative encoding intimin alpha/gamma chimera (Hartland et al., 2000)
pSWN01	pCVD438 derivative encoding intimin alpha/beta chimera (This study)
pCR [®] 4-TOPO	Cloning vector for sequencing PCR products in a single step (Invitrogen). Amp ^r , Kan ^r

Table 2.3 PCR Primers Used in This Study

<u>Name</u>	<u>Sequence</u>	<u>Source/Reference</u>	<u>Ann.Temp. (x) °C</u>	<u>Product Size bp</u>
EspP 5'	AAACAGCAGGCACCTTGAACG	(McNally et al., 2001)	57	301
EspP 3'	CCAGACAACCAATTGACAACC			
toxB 5'	GAGGCGCTGAAGATCAAACA	(McNally et al., 2001)	51	330
toxB 3'	GCGCACCTCCTGATAATACA			
Ehx 5'	ACGATGTGGTTTATTCTGGA	(Fagan et al., 1999)	53	165
Ehx 3'	CTTCACGTCACCATACATAT			
EAST 5'	CCATCAACACACAGTATATCCGA	(Yamamoto and Exheverria, 1996)	55	276
EAST 3'	GGTCGCCGAGTGACGGCTTTGT			
EspA 5'	CCGTTGTTAATGTGAGTGCG	(McNally et al., 2001)	56	299
EspA 3'	CACGTCCTTGAGGAAGTTTGG			
EspB 5'	GCCTGCTGAATCTGATAGCT	(McNally et al., 2001)	56	211

Esp B 3'	CGATGGTTAATTCGCTTCG			
Stx 5'	GAAAGGAAATAATTTATATGT	(Bastian et al., 1998)	45	900
Stx 3'	TTTGATTGTTACAGTCAT			
Stx Phage 5'	CGGATGTCTGGGTTCTGTC	This Study	56	1119
Stx Phage 3'	GGTCTGACTGCACTACCGT			
eaef A 5'	GTCGTCGTTAAGTCGATGGA	This Study	56	926
eaef A 3'	GACCTGACCAAATGCCAGC			
eaef B 5'	TCGTACAGTTCAGGCCT	This Study	56	927
eaef B 3'	TTGTGCGCTTTGGCTTCCG			
eaef C 5'	GCTGCAGTCGAATCCTGGT	This Study	56	995
eaef C 3'	GATCGGTTGTTGCTTCAGTC			
eaef D 5'	GCGAAGGCAGATGGTTCTG	This Study	56	1013
eaef D 3'	GCACCTGTCTACGAGTTG			
eaef E 5'	CTGTTGATAAECTCGGGAGTG	This Study	56	?

eaεβ E 3'	CTGTCCCTGATGGTCGTCA			
intB	TAAGGATTTTGGGACCC	(AduBobbie et al., 1998)	55	600
intG	ACAAACTTTGGGATGTTT			564
intRu	TTTATTTGCAGCCCCCAT			

Table 2.4 Restriction Endonucleases Used in This Study

Endonuclease	NEBuffer	Incubation Temperature (°C)	Incubation Time (hours)	Recognition Site (5' to 3')	Recognition Site (3' to 5')
<i>Bsa</i> I	3	50	2	GGTCTC(N) ₁	CCAGAG(N) ₅
<i>Eco</i> RI	ANY	37	2	G*AATTC	CTTAA*G
<i>Hinc</i> II	3&BSA	37	2	GTPy*PuAC	CAPu*PyTG
<i>Sph</i> I	2	37	2	AGG*CCT	TCC*GGA
<i>xba</i> I	2&BSA	37	16	T*CTAGA	AGATC*T

3.1 Introduction

Bacterial adherence can be investigated by quantifying the ability of any organism to adhere to intestinal epithelia *in vitro*. Previous studies have utilized immortalized cell lines (Wainwright et al., 1992) and primary cell cultures (O'Neil-Peterson et al., 2001) to investigate *E. coli* O157:H7 adherence to cells in culture that represent intestinal epithelium. In this chapter the use of a technique known as an *in vitro* organ culture (IVOC) is utilized to provide an epithelial surface *in vitro* that closely represents that maintained by the host *in vivo*. The IVOC system has been used to examine A-E lesions formed by EPEC and EHEC on human intestinal epithelium (Hicks et al., 1998) (Phillips et al., 2004) and *E. coli* O157:H7 adherence to bovine intestinal epithelium (Phillips et al., 2004). The methodology described in this chapter was used as the foundation to develop the system for the experiments described in this chapter. The tissue handling and culture conditions were optimized and a quantitative method of assessing adherence levels was developed in the early stages of this program of research.

Bacterial Affinity to Bovine Intestinal Mucosa Examined by *In Vitro* Organ Culture

3.1 Introduction

Bacterial adherence can be investigated by quantifying the ability of any organism to adhere to intestinal epithelium *in vitro*. Several studies have utilised immortalised cell lines (Winsor et al., 1992) and primary cell cultures (Dibb-Fuller et al., 2001) to investigate *E. coli* O157:H7 adherence *in vitro* to surfaces that represent intestinal epithelium. In this chapter the use of a technique known as *in vitro* organ culture (IVOC) is utilised to provide an epithelial surface *in vitro* that closely represents that encountered by the bacteria *in vivo*. The IVOC system has been used to examine A/E lesion formation of EPEC and EHEC on human intestinal epithelium (Hicks et al., 1998) (Phillips et al., 2000) and *E. coli* O157:H7 on bovine intestinal epithelium (Phillips et al., 2000) (Baehler and Moxley, 2000). The methodology described in these studies was used as the foundation to develop the system for the experiments described in this chapter. The tissue handling and culture conditions were optimised and a quantitative method of assessing adherence levels was developed in the early stages of this programme of research.

One of the main experiments examined strains that varied in their ability to secrete certain *LEE* encoded proteins in permissive *in vitro* conditions (McNally et al., 2001). A second major experiment examined the impact of intimin type in two different background strains, an *E. coli* O157:H7 human strain (ZAP 201) and an *E. coli* O26:NM isolated from a diarrhoeic calf (ZAP 21). In both experiments mucosa from four different intestinal sites were used to determine if the strains exhibited a tropism for any region of the GIT. It was anticipated that results from this work would provide valuable information for the calf model described in later chapters both in terms of which factors should be examined, by the use of deletion mutants, and by determining which regions of the GIT the bacterium was most likely to be interacting with.

3.2 Results

3.2.1 Early Studies

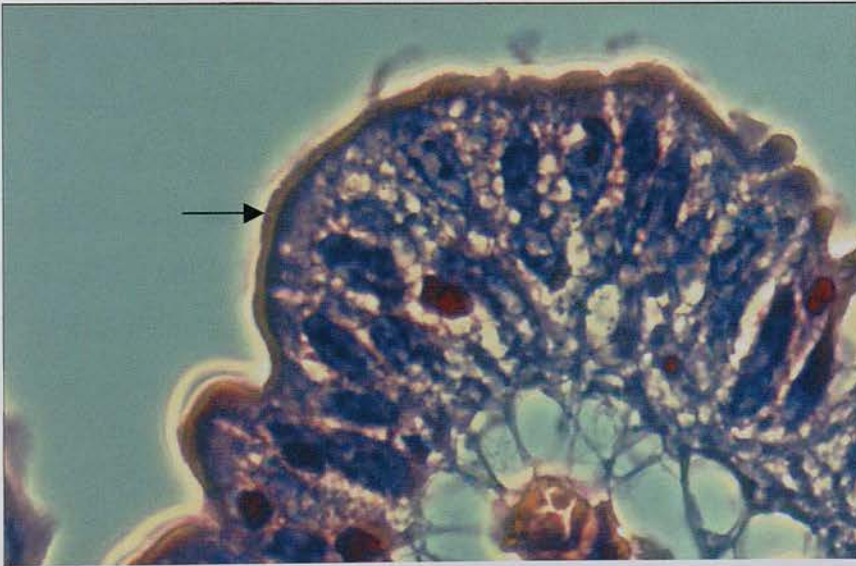
Early attempts at IVOC made use of intestinal mucosa taken from sheep following their use in Pasteurellosis experiments but problems with the tissue following culture included cryptal abscesses, death of sub-mucosal cells and autolysis of epithelium. Some sections contained large numbers of bacteria with some tissue invasion, as determined by Gram staining. It was possible that these animals were suffering from low grade or subclinical infections with common sheep enteric pathogens such as bacteria, helminths or coccidia that were compromising the intestinal epithelium in some way. In an attempt to alleviate the problem of pre-existing infections without resorting to antimicrobials, neonatal calves were sourced and tissue removed immediately post-mortem. With each attempt the logistics were improved to minimise the delay between death and establishment of the cultures within the laboratory.

Apart from the lack of contaminating bacteria, the neonatal calf IVOCs suffered from many of the same problems as the earlier sheep IVOC although the tissue was generally in better condition. Up until this point the tissue had been transported in culture medium at 37°C, a method advocated by Phillips *et al* who had access to human mucosal biopsies that were placed immediately into pre-warmed culture medium and were quickly transported to a lab within the same building. When obtaining tissue from freshly killed animals within a post-mortem room, there was an inevitable delay between death and placing the tissue into culture medium due to the time required for evisceration and dissection. Tissue was then transported to a laboratory and would have invariably experienced a drop in temperature despite attempts to insulate the transport vessels, especially as these attempts were performed in winter. By the fourth attempt the procedure was made more efficient and the cultures were set up inside a walk-in incubator at 37°C to ensure a rapid return to body temperature. Even when bacteria were added to the tissues at the commencement of culture, a proportion of the epithelium was in good condition at 0, 4 and 8 hours as determined by the presence of an intact brush border (fig. 3.1). However many areas of each sample lacked epithelium or the epithelium was autolytic or sloughing. In addition many test bacteria were present on the exposed

sub-mucosal tissue and where the epithelium was compromised. For the purposes of assessing epithelial adherent bacteria therefore, only bacteria that were definitely adherent to the surface of intact epithelium were included in the count data.

Figure 3.1 Bovine Peyer's Patch Mucosa Following 8 hours of IVOC
Demonstrating Intact Brush Border

A portion of intestinal epithelium following IVOC stained by H&E and photographed using a 40x objective. An intact brush border, more apparent when viewed through the eye-pieces, has been highlighted by phase contrast and appears as a red edge (arrow).



3.2.2 Assessment of Different Tissue Transport Conditions

Neonatal calves brought with them various logistical problems stemming from the fact that their presence on any particular day could not be assured. In addition the epidemiological observation that weaned calves have the highest prevalence of *E. coli* O157:H7 (Cobbold and Desmarchelier, 2000; Van Donkersgoed et al., 1999), together with the atypical pathology induced by this organism in neonatal calves suggested that there may be possible differences between these age groups that may be related to a tissue specific factor. It was therefore decided to rear calves to 12 weeks of age prior to performing the IVOC experiments. In order to verify that tissue from this age of animal was suitable for IVOC and to assess the impact of

various methodologies, an individual was sampled under terminal anaesthesia. The tissue was fixed on return to the lab and assessed by haematoxylin and eosin staining. No difference was observed between tissue taken under terminal anaesthesia and tissue taken immediately post-mortem, although in the latter case the tissue was still subjected to a delay prior to its addition to the transport media. Four different transport media were assessed, all IVOC media but either chilled (on ice) or at 37°C and either pre-oxygenated or not. The tissue transported at 37°C was superior to the tissue transported on ice in that epithelium in the latter exhibited distinctive patterns in the cytoplasm, possibly the result of ice crystal formation as the epithelial surface would have been separated from the ice by only a thin layer of plastic. Subsequent transport in pre-chilled media for an extended duration (see later) did not reproduce this effect. The possible deleterious effect of anoxia in tissue no longer perfused with blood is dealt with by using an oxygenated atmosphere during the culture conditions. In order to provide oxygen during transport 95% oxygen was bubbled through a column of chilled culture medium for several minutes. No difference was apparent between tissue transported in oxygenated or standard media but the former was used in subsequent IVOC experiments as a precaution due to the extended transport period imposed (see later).

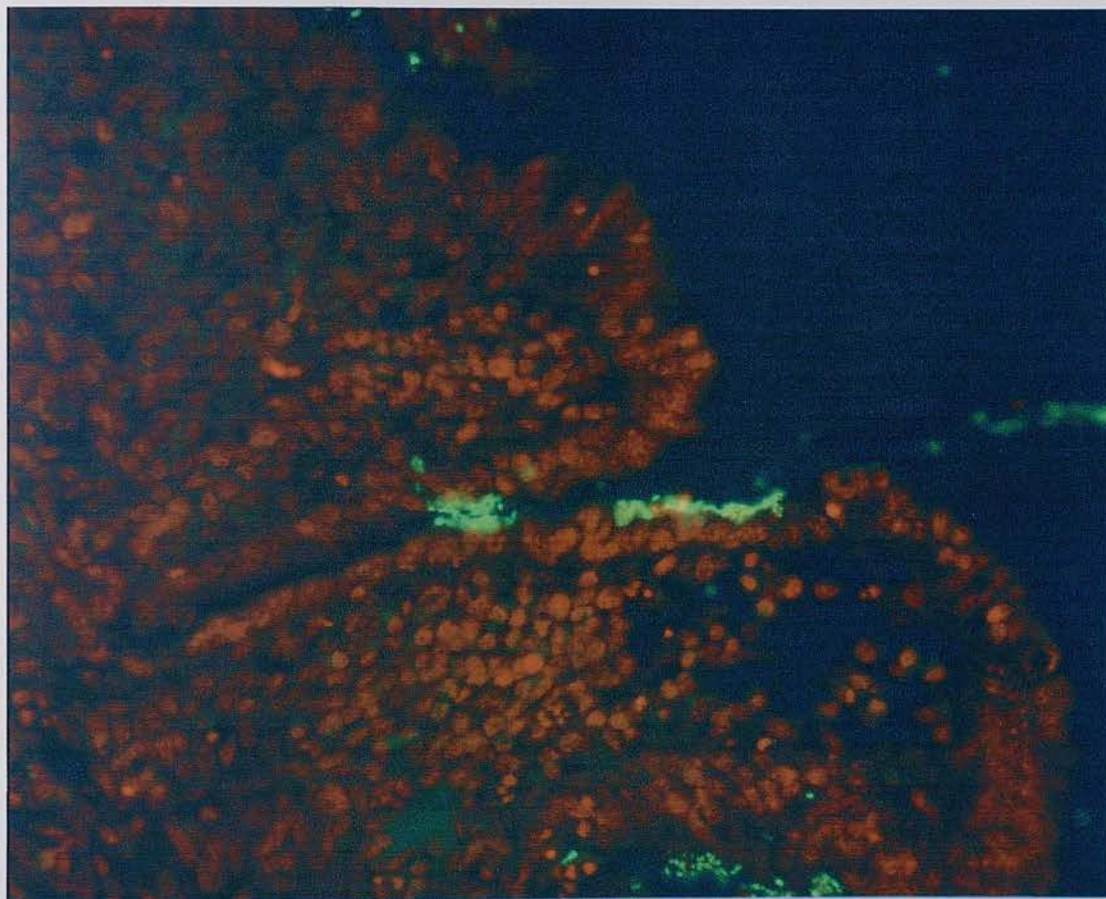
3.2.3 Development of IVOC as a Bacterial Affinity Assay

Previous IVOCs had added *E. coli* O157:H7 and visualised the bacteria by anti-LPS (lipopolysaccharide) immunofluorescence of paraffin embedded sections and by electron microscopy. Transmission electron microscopy only allows very small regions of tissue to be assessed, although it can determine whether bacteria are intimately attached, whereas scanning electron microscopy (SEM) does not allow assessment of the condition of the cells to which bacteria are adhering. In both cases the identity of any bacteria seen as the inoculated strain could only be made by the use of immunogold labelling which would have been time consuming and difficult to both develop and perform on a large scale. Immunofluorescence of multiple sections was therefore the preferred method as it allowed assessment of tissue quality, easy detection and identification of the test bacteria and was relatively quick and easy to process.

In order to quantify adherence on IVOC there are several problems. Firstly the numbers of adherent bacteria were low with many fields of intact epithelium containing no bacteria. Secondly there were occasional clusters of bacteria that may have been intimately attached microcolonies but could equally be a site where the loss of an enterocyte has exposed the underlying interstitial tissue. Figure 3.2 illustrates putative A/E microcolonies on a section of Peyer's patch epithelium following IVOC with ZAP 46. Additionally the large numbers of individual bacteria within such a colony may have a disproportional effect on the level of adherence. It was therefore decided to record a cluster of bacteria as a single adherence event. If a significant proportion of these were intimately attached then removal of a factor essential for the A/E phenotype, e.g. intimin, would reduce the levels of adherence. Finally there was a requirement to quantify the surface area of intact epithelium to which the bacteria could attach to allow direct comparison between samples. Because the epithelium contained some areas of autolytic or sloughing cells so only those microscope fields (as viewed with a 40x objective) containing intact epithelium were included in the adherence assay. Bacteria adherent to sloughed cells were excluded from the assay. Since the vast majority of fields contained either zero, one or two test bacteria, it was more efficient and less likely to be skewed by clusters of bacteria or bacteria that were not in fact adhering to enterocytes, if the adherence was expressed as "the proportion of fields containing intact epithelium that contained one or more test bacteria adhering to the epithelial surface". Strictly, this methodology measures affinity for the epithelium rather than adherence since it does not determine any specific adherence mechanism. Comparison of appropriate strains however has the potential to determine the effect of any putative adherence factor.

Figure 3.2 ZAP 46 Forming Putative A/E Lesions on Bovine Peyer's Patch Following 8 hours of IVOC

Foci of adherent *E. coli* O157:H7 (green) on Peyer's patch epithelium following 8 hours of culture (40x objective). The nuclei within the tissue are stained red with propidium iodide.



3.2.4 Tissue Tropism of *E. coli* O157:H7 Isolates Relative to Other Strains

In order to evaluate the above affinity assay for *E. coli* O157:H7 isolates, a Stx negative isolate (ZAP 193) as well as a Stx positive isolate (ZAP 3) were applied to IVOCs. At this time the procedures for containment level 3 work were being established. Prior to the use of ZAP 3 all IVOC work had been performed with Stx negative strains. As controls a bacterium was required with a corresponding antibody for immunofluorescence. Ideally other *E. coli* strains would have been

preferred as controls but the conventional negative control *E. coli*, K12 derivatives, are rough mutants and thus an anti-LPS antibody is not available. A negative control ideally contains no known factor that mediates epithelial adherence or invasion and the one that was selected was *Listeria innocua*, which is commonly used as a negative control with which to compare other *Listeria* spp. As a positive control the bovine AEEC strain O80:NM was selected as this was known to colonise the intestinal epithelium of calves (Pearson et al., 1989) and importantly, unlike many other bovine AEEC isolates, does not contain Stx.

The aforementioned protocol for quantifying affinity levels was applied to IVOC using tissue derived from two 12 week-old calves per test strain and the data analysed using the generalised linear mixed model. Tissue from five sites in the intestine, Peyer's patch, villous terminal ileum, proximal colon, spiral colon and rectum were cultured each time. Affinity levels, expressed as the proportion of fields containing epithelial adherent bacteria, were determined for each strain across all tissues. Although the negative control strain, *Listeria innocua*, exhibited very low levels of affinity as expected, there were no differences between the test strains and the positive control strain over all the samples analysed collectively. A limitation of this enumeration protocol is that any microcolonies are not incorporated into the data, other than as a single adherence event, so it was possible that AEEC O80:NM possessed an enhanced ability to form A/E lesions on the tissues tested.

Comparing each strain on the different tissue types (fig. 3.3) revealed a possible affinity for Peyer's patch epithelium for one of the O157 strains (ZAP 3) and the positive control strain (O80:NM), the former also had a higher affinity for the ileal epithelium compared to the large intestinal sites. One possible explanation for this result is that a proportion of Peyer's patch epithelium is comprised of follicle-associated epithelium (FAE) and as such functions as a sampling site for particulate antigenic material such as bacteria. The observed affinity could therefore have been non-specific, a theory consistent with the fact that the two strains displaying this tropism were of different serotypes. This observation was however interesting in the light of the Peyer's patch FAE restricted tropism reported for *E. coli* O157:H7 on human IVOC (Fitzhenry et al., 2002) (Phillips and Frankel, 2000) (Phillips et al., 2000).

In this study, no differentiation was made between FAE and villous epithelium within the samples taken from the Peyer's patch. Justification for this comes from the fact that after 8 hours of IVOC culture this distinction was difficult to make owing to the apparent collapse of villi and the widespread distribution of lymphoid follicles in the underlying tissue. The bacterial counts were therefore made over the entire sections on the assumption that a reasonable proportion of the epithelium was comprised of FAE. This assumption was based on the high density of follicles and the presence of short structures containing lymphoreticular cells similar to those described in neonatal calf Peyer's patch (Torres-Medina, 1981) (fig. 3.4).

Figure 3.3 Affinity Levels of Different *E. coli* Strains to Bovine IVOC

Mean levels of adherence determined by the proportion of fields containing epithelial adherent bacteria. Error bars indicate upper and lower 95% confidence intervals. The first graph illustrates the results of each strain over all tissue sites examined. The other three graphs illustrate each strain on each tissue type (PP – Peyer's patch, prox. colon - proximal colon, sp. colon – spiral colon).

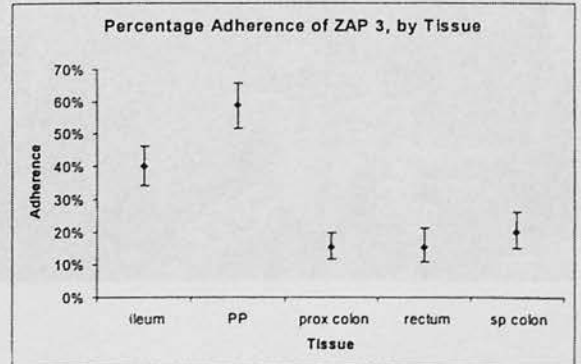
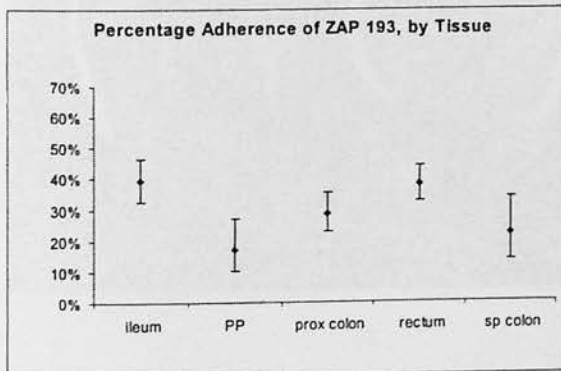
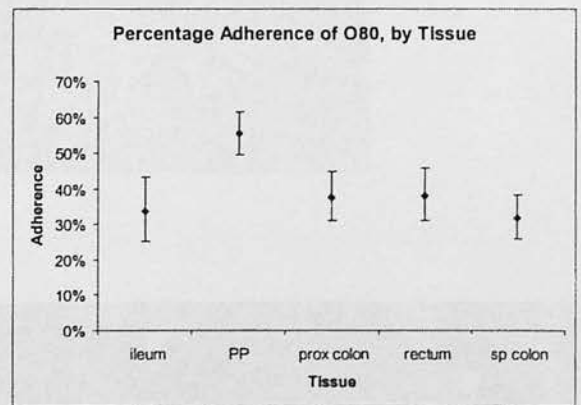
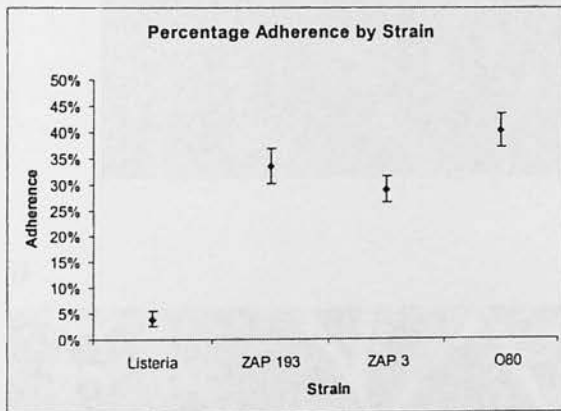
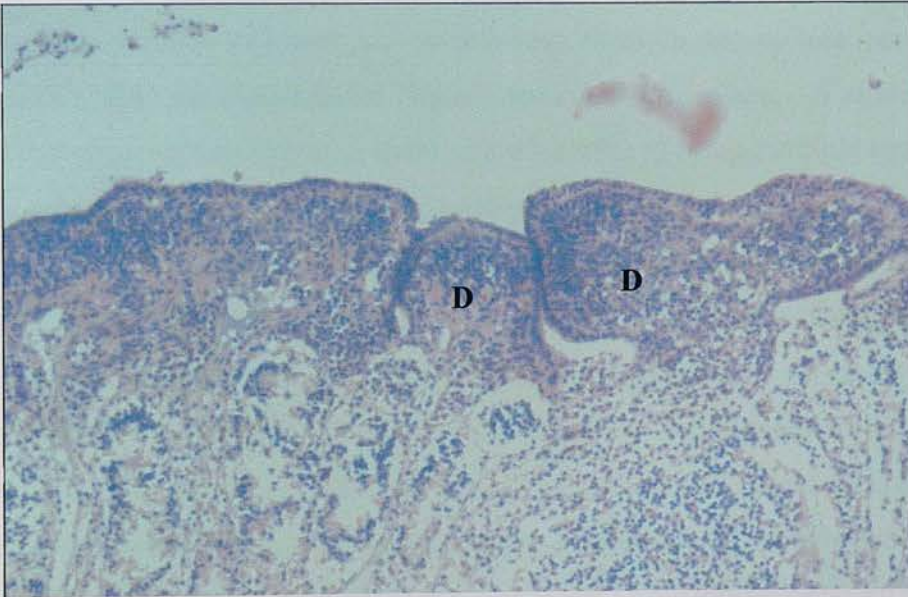


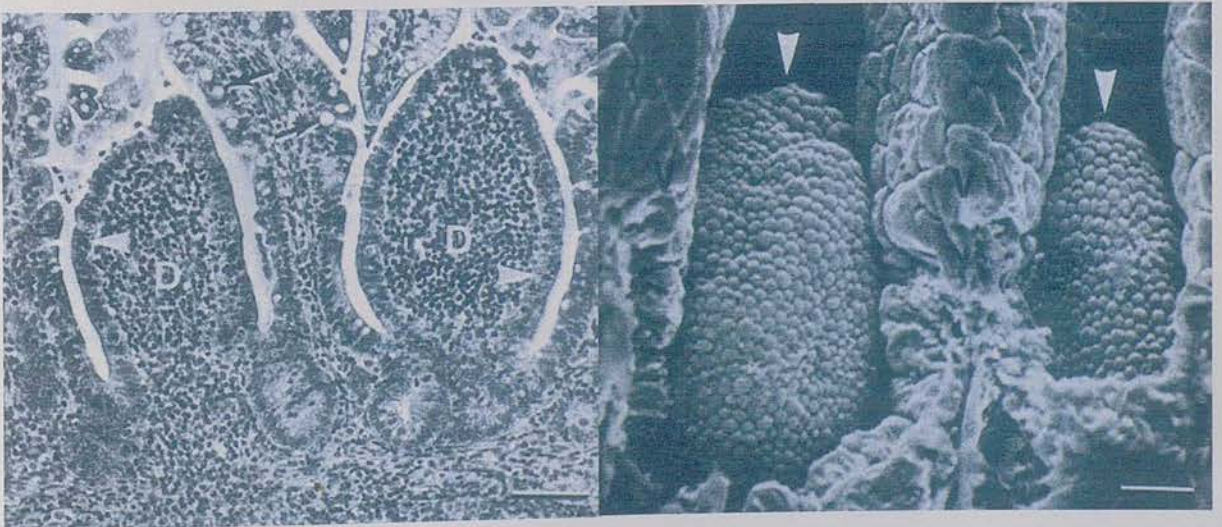
Fig 3.4 Peyer's Patch Following 8 hours of IVOC (Fig 3.4 A) and from a Neonatal Calf (Fig 3.4 B)

Distinctive domes (D) on a H&E stained section (this study) and a scanning electron micrograph from Torres-Medina (Torres-Medina, 1981). The presence of lymphocyte populations immediately below the epithelium characterise this as FAE. The structures have "collapsed" slightly during the 8 hour culture *in vitro*, a feature also observed in villous ileum where the villi were present but were no longer upright and finger-like.

A



B



3.2.5 An Improved Experimental Design

Although the ability of this methodology to identify differences in affinity levels between strains and different tissue types had been demonstrated, several areas for improvement were identified and incorporated into future IVOC experiments. These included the need to allow for between-animal variation, which could be done by testing all the bacterial strains to be examined on tissue from each site within each animal. Another was to examine a larger number of strains and to examine each strain on multiple IVOCs of tissue derived from the same site and animal. All these experimental improvements however were mutually exclusive, due to the limited number of cultures that can be established simultaneously, in turn due to the delay of preparing the mucosal explants and establishing them in the culture conditions, during which time additional tissue degradation could take place. A strategy was devised that allowed a compromise between the benefits of using multiple strains and multiple animals while still assessing the affinity to different tissue types. Use of a “balanced factorial in incomplete block design” (Mead, 1990) permitted the omission of a proportion of strain/site combinations thus reducing the number of cultures to be set up simultaneously to a manageable number. When repeated on tissue from a number of different calves with a different selection of tissue/strain combinations each time, it was possible to derive, using the generalised linear mixed model (Brown and Prescott, 1999), an estimation of the inherent variation between animals and between sections and fields of the same samples. Interactions between site and strain could then be established along with a measurement of statistical significance of any differences observed. This approach was applied to the subsequent experiments.

3.2.6 Tissue Tropism of *E. coli* O157:H7 Isolates

In an attempt to detect any possible tropism of *E. coli* O157:H7 for a region of the bovine intestinal tract, the affinity of five wild type isolates (ZAP 3, 26, 41, 46 and 58) were compared on mucosal explants of spiral colon, pre-pelvic rectum, ileal Peyer’s patch and the villous epithelium of the terminal ileum. In total, 6022 microscope fields were examined from the four sites collected from six different animals. A factorial design was utilised to enable all the combinations of factors to

be tested on a manageable number of explants per animal. To examine for any non-strain specific tropism at each of the sites the results from all strains were analysed collectively (fig. 3.5).

There was evidence of slightly lower adhesion in the pre-pelvic rectum with no significant differences apparent between any of the other sites. However, this behaviour was not apparent in all strains when examined individually. A summary of the estimated proportion of fields containing adherent bacteria on the epithelial surface at each site is shown in table 3.1. Although several individual strain/site combinations deviated significantly from the sample mean there was no consistent tissue tropism across all the strains. ZAP 58 shows statistically significantly lower tropism for both Peyer's patch and rectum than in the other two sites ($p < 0.001$). ZAP 3 shows lower tropism for the colon, this being statistically significant compared to the other sites ($p = 0.02$). ZAP 26 shows a significantly lower tropism for Peyer's patch ($p = 0.005$) and higher tropism in the colon ($p < 0.001$) when compared with the other sites. ZAP 41 and ZAP 46 show statistically significantly lower tropism in rectum ($p < 0.001$) and colon ($p < 0.001$) tissues respectively. The affinity levels on each tissue type were also analysed for each strain. Although each strain demonstrated significantly higher or lower affinity to one or more tissue types there was no obvious pattern and these differences cancelled one another out when analysed collectively, with the possible exception of the mid-rectum samples, on which there was a slightly lower affinity level.

Figure 3.5 Mean Affinity of All Wild Type Strains at Each Intestinal Site

Error bars indicate upper and lower 95% confidence intervals. TI – Terminal Ileum, PP – Peyer’s Patch, COL – Spiral Colon, REC – Mid Rectum

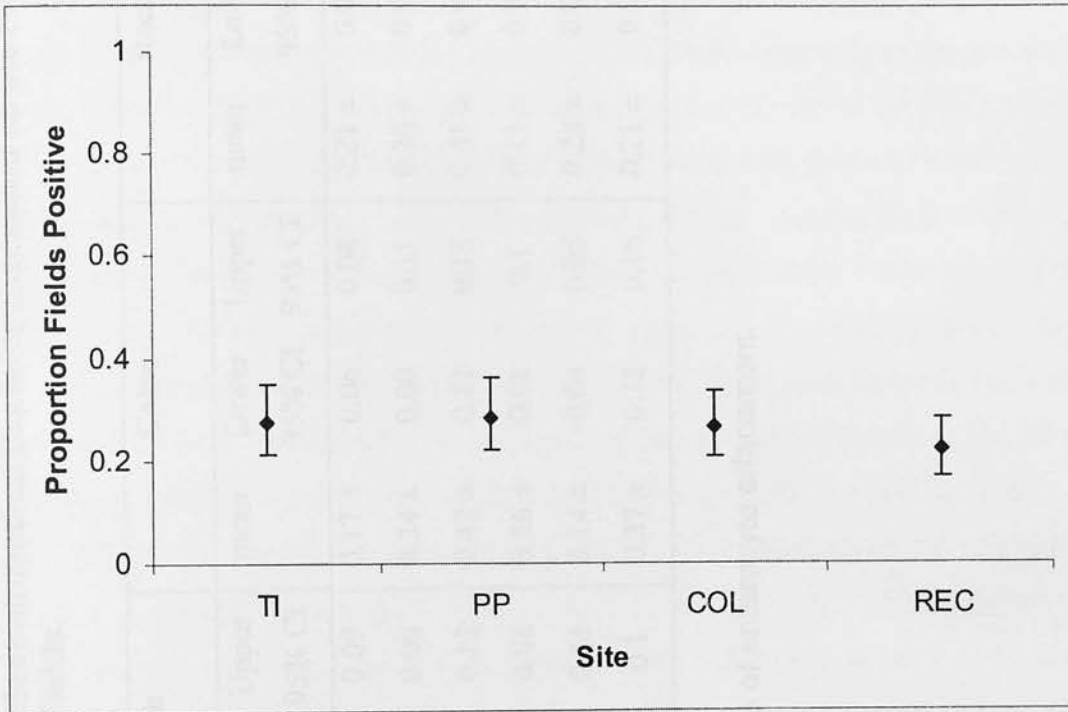


Table 3.1 Affinity of Different *E. coli* O157 Strains for Bovine Gastrointestinal Explants Expressed as the Proportion of Positive Fields.

Strain	Site	Terminal Ileum			Peyer's patch			Colon			Rectum		
		mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI
3	(high Esp)	0.30 ±	0.09	0.11	0.25 ±	0.07	0.09	0.17 ±	0.06	0.08	0.21 ±	0.06	0.08
26	(high Esp)	0.24 ±	0.06	0.08	0.14 ±	0.04	0.06	0.34 ±	0.09	0.11	0.26 ±	0.07	0.1
27	LEE -ve	0.21 ±	0.05	0.07	0.59 ±	0.12	0.12	0.42 ±	0.11	0.13	0.31 ±	0.09	0.12
41	(low Esp)	0.22 ±	0.06	0.08	0.26 ±	0.07	0.08	0.26 ±	0.08	0.1	0.11 ±	0.03	0.05
46	(low Esp)	0.36 ±	0.09	0.11	0.31 ±	0.09	0.11	0.14 ±	0.04	0.05	0.28 ±	0.08	0.1
58	(high Esp)	0.36 ±	0.1	0.12	0.27 ±	0.08	0.1	0.37 ±	0.12	0.16	0.21 ±	0.06	0.08

CI - confidence interval; Esp - *E. coli* secreted protein; LEE - locus of enterocyte effacement.

3.2.7 Effect of *LEE* Protein Secretion Status on Tissue Tropism

The *LEE* protein secretion status of the study strains in MEM-HEPES was confirmed (described in detail below), with ZAP 3, 26, and 58 secreting higher levels than ZAP 41 and 46. The binding data for the high secreting strains was combined and compared with the combined data for the two low secreting strains (fig. 3.6 and Table 3.1). There was evidence of significant interactions between protein status and tissue affinity. High secreting strains showed significantly lower ($p=0.04$) affinity for Peyer's patch, but significantly higher affinity for colon ($p<0.001$) and rectum ($p=0.006$) tissues, compared with the low secreting strains. Among the low secretors the levels of adhesion in the Peyer's patch and terminal ileum were significantly higher ($p<0.001$) than in the colon and rectum. Among high secreting strains, there were no appreciable differences between the affinity levels seen between the colon and terminal ileum, and between the rectum and Peyer's patch. However, the levels of affinity in the former sites were significantly higher ($p<0.001$) than in the latter.

Significant differences between the groups were detected on Peyer's patch, colon and rectum, although, as can be seen in fig. 3.6, the magnitude of the difference was not large, the largest occurring on the colon (low secretors 0.19, high secretors 0.28). The high secretors adhered significantly better than the low secretors on both the large intestinal sites but the converse was true on the Peyer's patch tissue. These results imply that *LEE*-mediated adherence contributed significantly to the affinity observed on the large intestinal tissue. This is consistent with certain studies in which *E. coli* O157:H7 colonised the large intestine at higher levels than the small intestine in experimentally challenged calves. *LEE* encoded factors may have been responsible for this tropism, in which case strains with higher levels of *LEE* expression would be expected to adhere to large intestinal tissue better than low secreting strains. No difference in affinity between high and low secretors was observed on the terminal ileum suggesting that *LEE* secretion status was not contributing to a significant portion of the affinity observed at this site.

It is possible that this adherence assay, which did not determine whether bacteria were adhering via intimate attachment, included a large proportion of non-intimately attaching bacteria in the data, which diluted any impact that *LEE*-secretion status may have had. The fact that the *LEE* negative deleted ZAP 27 adhered as well

as its parent strain, ZAP 26, to all the tissue types examined (with the exception of Peyer's patch, see 3.2.10) is consistent with this. The limited number of strains used in this IVOC experiment, i.e. 2 low versus 3 high *LEE* secretors, may have resulted in factors other than the *LEE* protein secretion status contributing to the observed differences between strains. Performing the experiment on a larger number of strains or the use of isogenic low and high secretors may have alleviated this problem. Neither alternative was possible due to the limited number of IVOCs that could be set up simultaneously, in the case of the former, and our lack of understanding of the molecular basis for *LEE* protein secretion heterogeneity in the case of the latter. It may also have been possible that the culture conditions were not favourable for A/E lesion formation due to the oxygen-enriched atmosphere. Baehler and Moxley (Baehler and Moxley, 2000) showed that A/E lesion formation on bovine IVOC was improved without oxygen enrichment. However the tropism of *E. coli* O157:H7 for the terminal rectum observed *in vivo*, described in Chapter 5, provides a possible explanation for the apparently poor affinity to the tissues examined within these IVOC experiments.

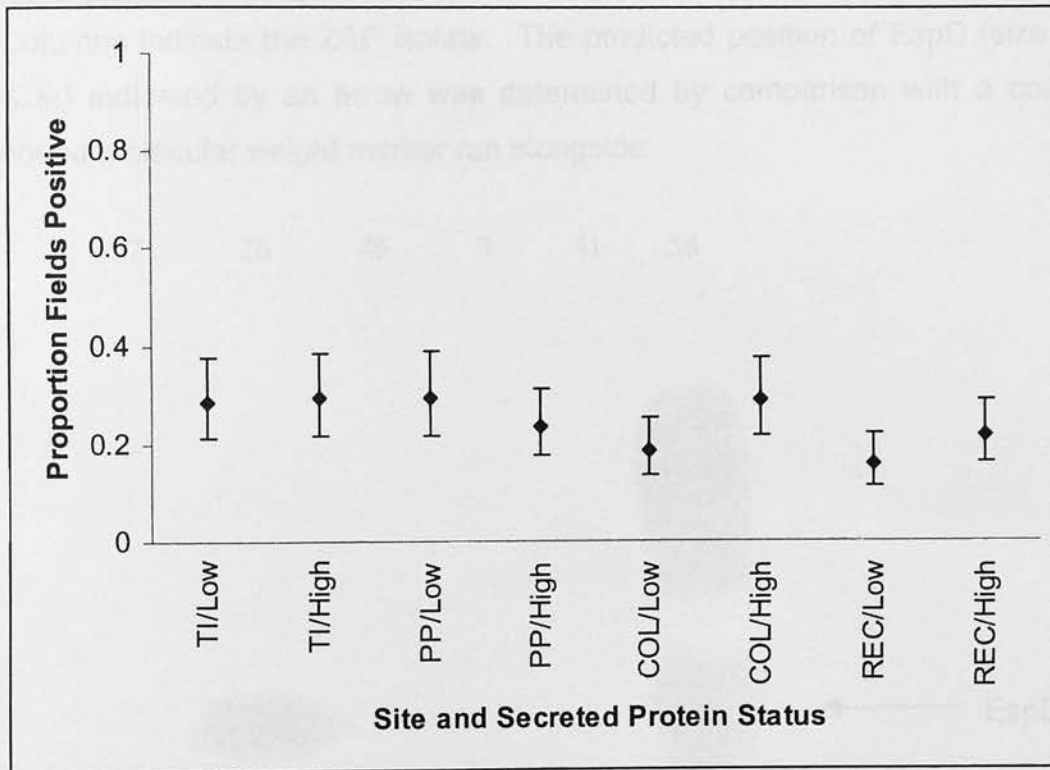


3.2.3 Homogeneity of *LEE* Protein Secretion Status by Western Blotting

First strains that varied in their ability to secrete *LEE* encoded proteins as determined by Mikhally *et al.* (2001) were selected for use in the IVOC affinity assay. To confirm their assigned protein status the strains were grown in M169-HEVES to mid-log phase and the culture supernatant subjected to TCA protein precipitation. The samples were processed with identical methods and run on the same SDS-PAGE gel for subsequent western blotting with anti-*hlyE* monoclonal antibody (fig 3.7). The results shown by Mikhally *et al.* to be high secretors produced bands of approximately 40 kDa of varying strength (3, 20 and 53) whereas the low secreting strains (4) had

fig. 3.6 Comparison of Affinity of High Secreting and Low Secreting Strains to Intestinal Explants from Each Site Examined

Error bars indicate upper and lower 95% confidence intervals. TI – Terminal Ileum, PP – Peyer's Patch, COL – Spiral Colon, REC – Mid Rectum. Low/High indicates Esp secretion levels in MEM-HEPES in mid-log phase.



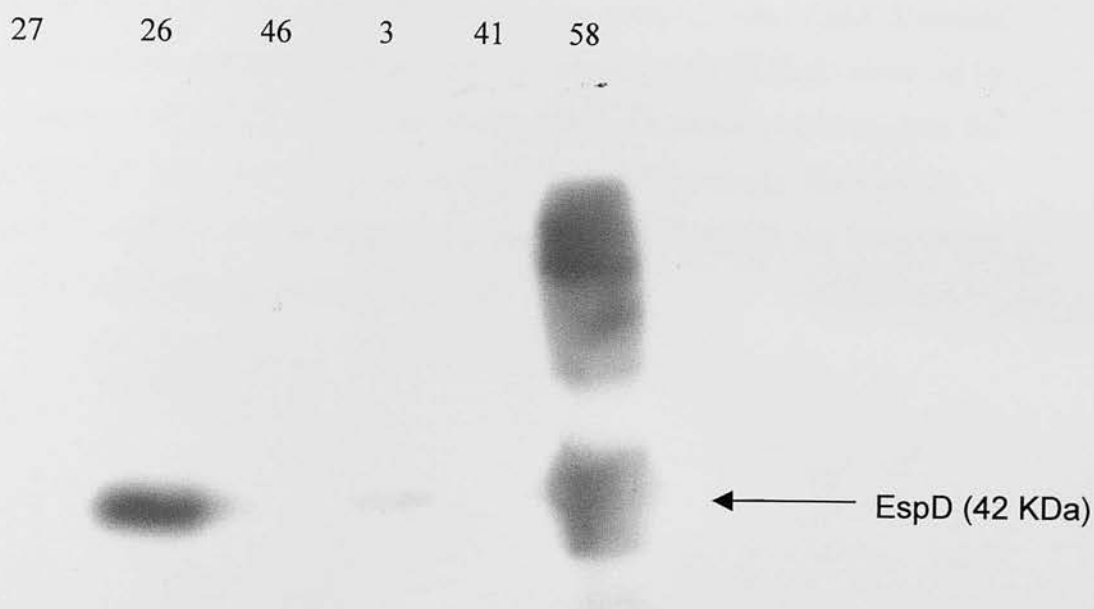
3.2.8 Demonstration of *LEE* Protein Secretion Status by Western Blotting

Five strains that varied in their ability to secrete *LEE* encoded proteins as determined by McNally et al. (2001) were selected for use in the IVOC affinity assay. To confirm their secreted protein status the strains were grown in MEM-HEPES to mid-log phase and the culture supernatant subjected to TCA protein precipitation. The samples were processed with identical methods and run on the same SDS-PAGE gel for subsequent western blotting with anti-EspD monoclonal antibody (fig 3.7). The strains shown by McNally *et al* to be high secretors produced bands of approximately 40 Kda of varying strength (3, 26 and 58) whereas the low secreting strains (41 and

46) did not produce visible bands. Previously, McNally *et al* showed that the low levels of EspD produced by these low secreting strains could be detected by co-precipitation with bovine serum albumin. This method produces far too strong a signal with the high secreting strains to allow direct comparison between all the strains.

Figure 3.7 Western Blot of Secreted EspD

Columns indicate the ZAP isolate. The predicted position of EspD (size 42 KDa) indicated by an arrow was determined by comparison with a colour coded molecular weight marker run alongside.



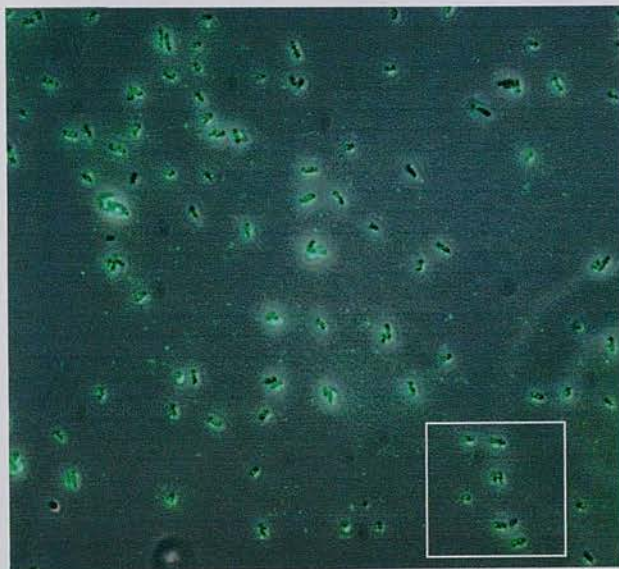
3.2.9 Demonstration of *LEE* Protein Secretion Status by Direct Immunofluorescent Labelling of EspA Filaments

A second method to confirm the *LEE* encoded protein expression levels within the test strains was to directly label EspA filaments and surface intimin by direct immunofluorescence. Strains were grown in the same conditions described for

secreted protein precipitation and labelled with antisera raised against EspA and intimin respectively (provided by Martin Woodward, VLA, Surrey). As shown in figure 3.8, EspA filaments could be visualised as fluorescent foci on the surface of bacteria. In contrast intimin was visualised within the membrane as a fluorescent halo, identical to the labelling produced by anti-LPS antibody labelling. Unlike the protein precipitation method that provides an indication of the average levels of protein produced across the entire population of bacteria within a culture, direct labelling permits observation of protein expression of individual bacteria. This approach led to the discovery that the levels of expressed protein was apparently a result of phase variation, with individual bacteria either expressing multiple filaments or none at all (fig. 3.8). The proportion of bacteria expressing either EspA filaments or intimin correlated with each other and with the level of secreted EspD observed by western blotting (table 3.2). This observation provides a major contribution to the understanding of *LEE* encoded protein expression. Importantly for this IVOC experiment, it confirms the heterogeneity of the strains selected in the factor being examined, i.e. *LEE* encoded protein expression.

Figure 3.8 Overlaid Phase Contrast and Indirect Immunofluorescent Micrographs of ZAP 198 Demonstrating Expression of EspA Filaments (x100 objective) (A) and a Small Area Enlarged (B)

A



B

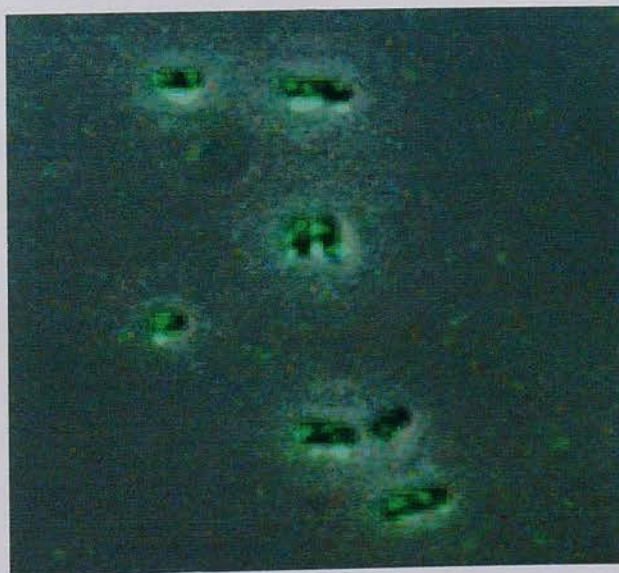


Table 3.2 EspD Secretion Levels and Proportion of Population Expressing EspA Filaments of a Variety of Strains Grown in Permissive Conditions

Strain	Serotype	Proportion With Filaments	EspD Secretion Status
ZAP 201	O157:H7	*****	ND
ZAP 202	O157:H7	*****	ND
ZAP 210	O157:H7	*****	ND
ZAP 204	O157:H7	*****	ND
ZAP 203	O157:H7	*****	ND
ZAP 21	O26:H-	*	ND
ZAP 25	O26:H-	*****	ND
ZAP 211	O26:H-	*****	ND
ZAP 212	O26:H-	****	ND
ZAP 27	O157:H7	0	Nil
ZAP 278	O157:H7	*	ND
ZAP 41	O157:H7	*	Very Low
ZAP 46	O157:H7	*	Low
ZAP 3	O157:H7	**	Low/Moderate
ZAP 26	O157:H7	***	Moderate
ZAP 58	O157:H7	***	Moderate
ZAP 193	O157:H7	*****	High
ZAP 195	O80:NM	**	ND
ZAP 196/198	O157:H7	*****	High

0 – No filaments labelled, * - <20%, ** - 20 to 40%, *** - 40 to 60%, **** - 60 to 80%, ***** - 80 to 100% estimated proportion of bacteria expressing filaments. EspD levels determined as described in 3.2.8 and classified according to the relative intensity of the band produced by Western blotting.

3.2.10 Comparison of Mucosal Affinity of a *LEE* Deleted Mutant with its Isogenic Parent

The affinity of ZAP 26 (EDL933) was compared with its *LEE*-deleted derivative, ZAP 27, to each tissue type (fig. 3.9 and table 3.1). The results show no significant difference on three of the tissue types: villous ileum ($p=0.36$), colon ($p=0.09$) and rectum ($p=0.28$). However, on Peyer's patch the *LEE* negative strain was present at the mucosal surface at significantly higher levels, approximately 4-fold, than the *LEE* positive parent strain ($p<0.001$). Examples of ZAP 27 affinity for this site are shown (fig. 3.10 (a), & (b)). Levels of affinity determined by the above method does not take into account fields containing multiple adherent bacteria, so the numbers of individual ZAP 26 and ZAP 27 were counted on the Peyer's patch mucosal surface. For ZAP 26 there were 88 bacteria in 248 microscope fields and for ZAP 27 there were 856 bacteria in 365 fields. Assuming the mean area of epithelial surface within fields was similar between the two groups, ZAP 27 adhered 6.6 times more effectively than ZAP 26 ($p<0.001$) to this epithelium type. The affinity of ZAP 27 to all tissue types examined was not significantly lower than the other strains with the possible exception of terminal ileum. Either *LEE* mediated adherence was not contributing to the observed affinity levels or the absence of the *LEE* in ZAP 27 was being compensated for by another factor.

Because ZAP 26 and 27 are isogenic, it is likely that the observed difference was a result of the absence of the *LEE*. It was possible that the *LEE* was repressing the expression of an alternative adherence factor(s). The result of a paper by Elliot *et al* (Elliott *et al.*, 2000) provides evidence for this. Their work demonstrated that the deletion of the *LEE* encoded regulator (*LER*) in *E. coli* O157:H7 results in the expression of fimbriae structurally similar to the long polar fimbriae (LPF) of *Salmonella typhimurium* (Baumler and Heffron, 1995). In ZAP 27 the *LER* is deleted along with the *LEE* and any repressive effects are therefore also absent. The possibility that ZAP 27 expresses fimbriae was investigated using transmission electron microscopy of bacteria negatively stained with phosphotungstic acid. Within a sample of ZAP 27, two bacteria possessed long straight fimbriae located at the poles (fig. 3.11), structurally resembling LPF of *S. typhimurium*. These fimbriae

were not seen in the other strains examined, although numerous short structures, presumably EspA filaments, were seen in the other strains examined (3, 26, 41, 46 and 58). The hypothesis that LPF upregulation was the reason for the enhanced affinity of ZAP 27 on Peyer's patch epithelium is strengthened by the fact that *S. typhimurium* LPF mediates a tropism for the murine Peyer's patch.

In wild type strains LPF may be optimally expressed in conditions that repress *LER* expression and vice versa. If this were the case then the growth conditions used in this study were clearly inappropriate for optimal LPF expression. One possibility arising from this hypothesis is that the enhanced affinity of low Esp secretors over high Esp secretors on the Peyer's patch occurred as a result of lower *LER* expression. The consequent increase in LPF expression mediating a slightly higher affinity to the Peyer's patch, just as it did for ZAP 27. However the *LER* levels in high and low secretors are identical in mid-log growth in MEM HEPES, as determined by reporter-gene fusions (Roe *et al.*, submitted for publication).

An observation made during the TEM analysis of the high and low secreting strains was that the proportion of bacteria expressing structures assumed to be EspA filaments did not correlate well with the reported *LEE* secreted protein status. This may have been a result of the small numbers of bacteria within the samples. However, inconsistent Esp levels in precipitated supernatants occurred whilst generating the blots for this piece of work. It is possible that the proportion of phase on bacteria varies considerably between different cultures of the same strain. Future work with such strains must include an analysis of the proportion of Esp producing bacteria within each individual bacterial culture. However it has been subsequently determined that much of this between-culture variation was due to inconsistent levels of glucose within the culture media used to grow the strains (MEM HEPES). Additional variation was also thought to arise from inconsistent levels of trace elements and the addition of MOPS trace elements (Neidhardt *et al.*, 1974) is required for optimal *LEE* expression.

Figure 3.9 Comparison of ZAP 26 and Its Isogenic *LEE* Deleted Derivative, ZAP 27, on Each Tissue Site Examined

Error bars indicate upper and lower 95% confidence intervals. TI – Terminal Ileum, PP – Peyer’s Patch, COL – Spiral Colon, REC – Mid Rectum.

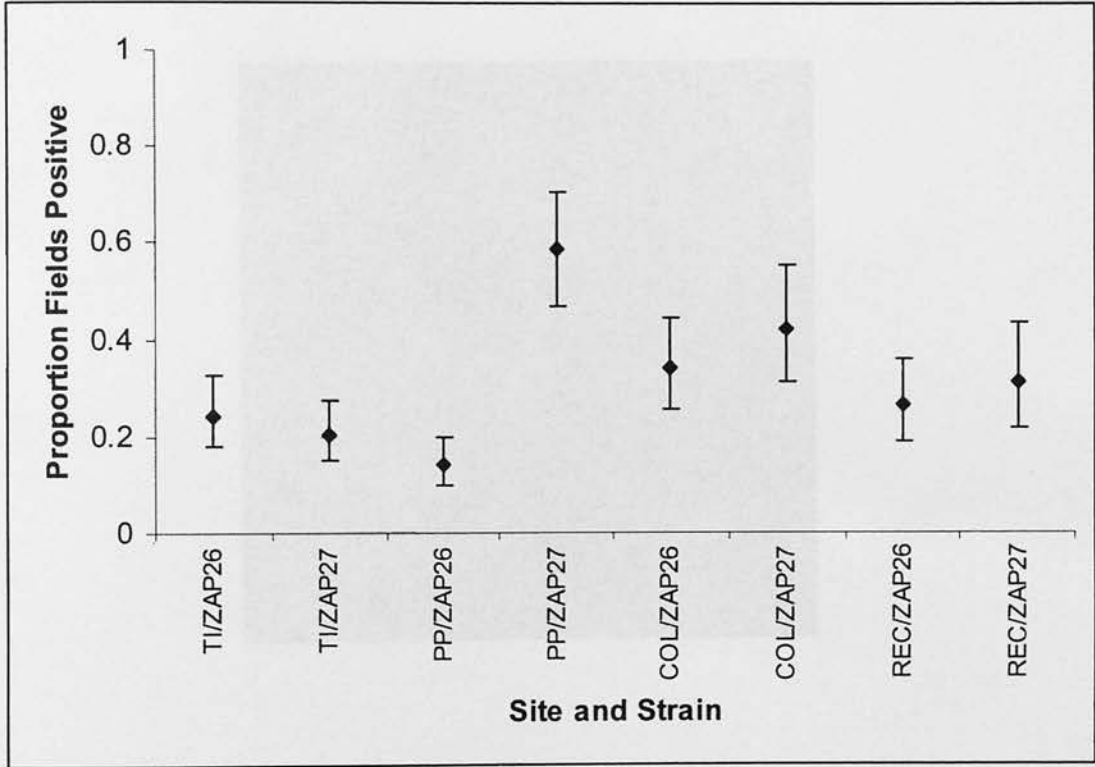
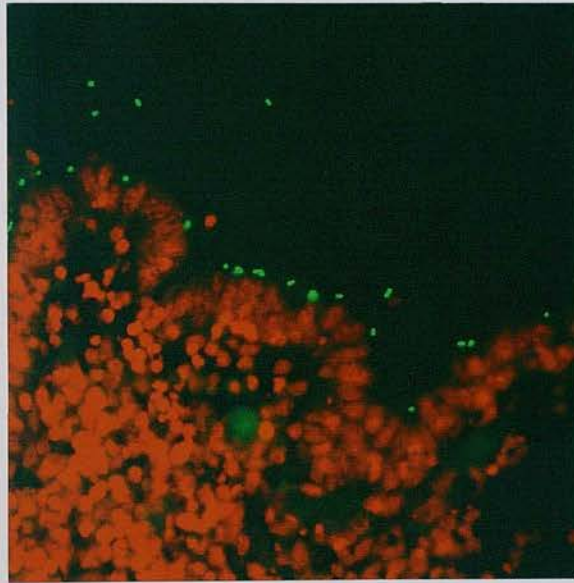


Figure 3.10 *E. coli* O157:H7 EDL933 Δ LEE (ZAP 27) at the Mucosal Surface of Bovine Peyer's Patch Following 8 hours of IVOC

Immunofluorescence of *E. coli* O157:H7 (green) (40x objective) with nuclei of tissue cells stained with propidium iodide (red) (A). TEM of a bacterium present on the same sample (10000x) (B).

A



B



3.2.11 Demonstration of Putative LPF and EspA Filaments by Transmission

Electron Microscopy

To provide a possible explanation for the enhanced binding to Peyer's patch epithelium of the *LEE* negative strain all the strains used in this experiment were grown to mid-log phase in MEM-HEPES, immobilised on a carbon-formvar grid and negatively stained with phosphotungstic acid. Structures resembling the long polar fimbriae of *S. typhimurium* were present only on ZAP 27 (fig. 3.11). Structures that were probably EspA filaments were seen on a proportion of the other strains, with the exception of ZAP 46. The numbers of bacteria with each structure are summarised in table 3.3.

Table 3.3 Relative Numbers of Surface Organelles on Different *E. coli* O157:H7 Strains

Strain	ZAP 3	ZAP 26	ZAP 27	ZAP 41	ZAP 46	ZAP 58
Surface Organelle						
No structures visible	26	14	34	28	40	36
EspA	4	16	0	2	0	4
LPF	0	0	2	0	0	0
Flagella	0	0	0	2	0	1

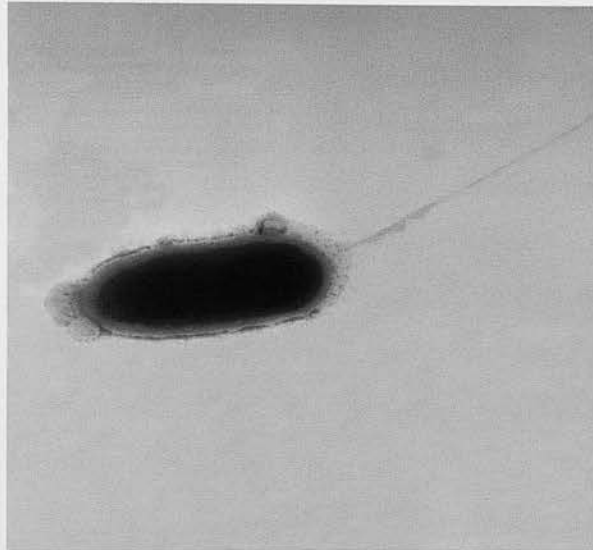
Figures indicate actual numbers counted;

EspA – *E. coli* secreted protein A;

LPF – Long polar fimbriae

Figure 3.11 Transmission Electron Micrograph of an LPF-Like Structure on ZAP 27

A fimbrial structure on the LEE negative *E. coli* O157:H7 can be seen emanating from the polar region (22000x). This structure extended for approximately 3 times the length of the bacterium and was approximately half the diameter of a flagellum.



3.2.12 Role of Intimin in Adherence to Bovine Intestinal Epithelium

Five serologically distinct intimin types have been published (AduBobie et al., 1998) (Oswald et al., 2000), with an additional 4 types deposited in GenBank. Intimin type has been shown to mediate tropism for different host and tissue types. Using human IVOC the tissue specificity of EPEC (throughout the small intestine) could be changed to that of *E. coli* O157:H7 (restricted to follicle associated epithelium), by deleting *eaeA* α and complementing with *eaeA* γ (Fitzhenry et al., 2002). On bovine IVOC, intimin γ expressing *E. coli* O157:H7 has previously been shown to form occasional A/E lesions on both bovine large intestine (Baehler and Moxley, 2000) and Peyer's patch (Phillips et al., 2000). Various non-O157 EHEC serotypes (Jenkins et al., 2002) colonise cattle and some have been isolated from diarrhoeic calves in which A/E lesions were detected (Hall et al., 1985; Schoonderwoerd et al.,

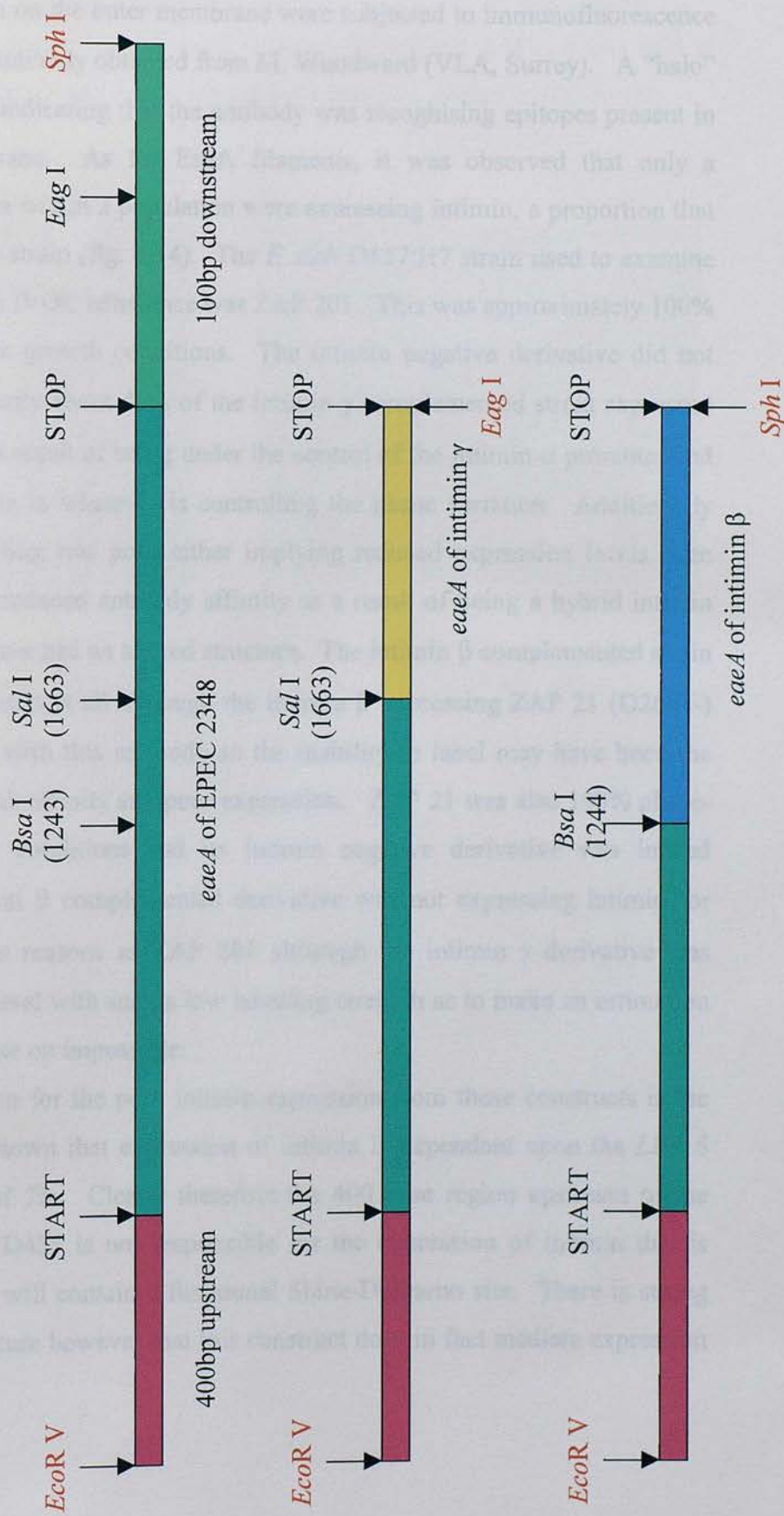
1988) (Pearson et al., 1999). Bovine AEEC mostly express intimin β , it is therefore a reasonable hypothesis that these strains can efficiently form A/E lesions throughout extensive regions of the bovine intestine and that this is dependant upon intimin type. The ability of two intimin types, γ from *E. coli* O157:H7 and β from *E. coli* O26:H-, to influence affinity to bovine intestinal epithelium was assessed using IVOC.

3.2.13 Generation of Intimin α / β Hybrid

E. coli O157:H7 85-170 (ZAP 201) and its intimin deleted derivative, 85-170 $\Delta eaeA$ (ZAP 202) were provided by G. Frankel (Imperial College, London). In addition, STEC O26:H- 413/89-1 strain (ZAP 21) and its intimin deleted derivative (ZAP 25) were provided by T. Chakraborty (Giessen University, Germany). A pACYC184 derivative contained the cloned *eaeA* gene, plus approximately 400 bp and 100 bp upstream and downstream respectively from the start and stop sites, encoding intimin α from a human EPEC isolate termed pCVD438 (Donnenberg and Kaper, 1991). The *N*-terminal 1158 bps, encoding the hypervariable region of *eaeA* that includes the Tir binding site (Luo et al., 2000) was replaced with the equivalent region of *eaeA* of *E. coli* O157:H7 85-170 (fig. 3.12 (b)) and designated pICC55. Because most bovine AEEC isolates contain intimin β whereas intimin α is typically expressed by human EPEC strains, for examining adherence to bovine intestinal epithelium it was thought more appropriate to generate a pCVD438 derivative containing the *N*-terminal 1576 bp of *eaeA* β from *E. coli* O26:H- (ZAP 21) designated pSWN01 (fig. 3.12 (c)). Both ZAP 202 and ZAP 25 were complimented with the plasmids pSWN01 (ZAP 210 and 211) and pICC55 (ZAP 204 and 212).

Figure 3.12 Schematic Representation of Cloned *eaeA* and Hybrid Derivatives

The various constructs cloned into pACYC184 indicating the start and stop codons and relevant restriction sites.



3.2.14 Demonstration of Intimin Expression

3.2.14.1 Demonstration of Intimin Expression by Indirect Immunofluorescence

Strains grown in permissive conditions (MEM-HEPES to mid-log phase) for expression of intimin on the outer membrane were subjected to immunofluorescence using anti-intimin γ antibody obtained from M. Woodward (VLA, Surrey). A "halo" effect was observed indicating that the antibody was recognising epitopes present in the bacterial membrane. As for EspA filaments, it was observed that only a proportion of bacteria within a population were expressing intimin, a proportion that varied from strain to strain (fig. 3.14). The *E. coli* O157:H7 strain used to examine the role of intimin on IVOC adherence was ZAP 201. This was approximately 100% phase on under these growth conditions. The intimin negative derivative did not label at all whereas only about 40% of the intimin γ complemented strain expressed intimin, possibly as a result of being under the control of the intimin α promoter and therefore less reactive to whatever is controlling the phase variation. Additionally the strength of labelling was poor either implying reduced expression levels even when phase on or a reduced antibody affinity as a result of being a hybrid intimin protein which may have had an altered structure. The intimin β complemented strain did not label for intimin at all although the intimin β expressing ZAP 21 (O26:H-) only reacted weakly with this antibody so the inability to label may have been the additive effect of weak affinity and poor expression. ZAP 21 was also 100% phase-on in these growth conditions and its intimin negative derivative was indeed negative. The intimin β complemented derivative was not expressing intimin for presumably the same reasons as ZAP 201 although the intimin γ derivative was expressing at a low level with such a low labelling strength as to make an estimation of the proportion phase on impossible.

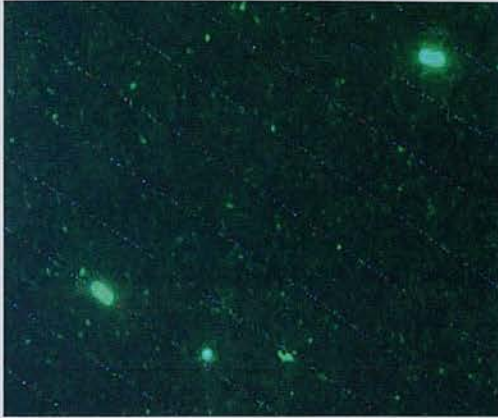
An explanation for the poor intimin expression from these constructs is the fact that it is now known that expression of intimin is dependant upon the *LEE 5* promoter upstream of *Tir*. Clearly therefore the 400 base region upstream of the cloned *eaeA* in pCVD438 is not responsible for the expression of intimin that is observed although it will contain a functional Shine-Dalgarno site. There is strong evidence in the literature however that this construct does in fact mediate expression

of intimin and this can be explained by a basal level of promoter activity from the pACYC184 derived plasmid.

Figure 3.13 Phase Variable Intimin Expression of ZAP 26 (EDL933)

Indirect immunofluorescent labelling of intimin (green) on ZAP 26 (A) and a phase contrast image of the same field demonstrating phase variation (B).

A



B



Table 3.4 Intimin Expression in Various Strains

Strain	Serotype	Intimin 280 Type	Proportion Labelled	Labelling Strength
ZAP 201	O157:H7	γ	*****	strong
ZAP 202	O157:H7	Negative	0	
ZAP 210	O157:H7	β	0	
ZAP 204	O157:H7	γ	***	weak
ZAP 203	O157:H7	α	0	
ZAP 21	O26:H-	β	*****	weak
ZAP 25	O26:H-	Negative	0	
ZAP 211	O26:H-	β	0	
ZAP 212	O26:H-	γ	ND	very weak
ZAP 27	O157:H7	Negative	0	
ZAP 278	O157:H7	γ	*	weak
ZAP 41	O157:H7	γ	*	moderate
ZAP 46	O157:H7	γ	*	moderate
ZAP 3	O157:H7	γ	*	moderate
ZAP 26	O157:H7	γ	*	moderate
ZAP 58	O157:H7	γ	***	moderate
ZAP 193	O157:H7	γ	*****	strong
ZAP 194	O80:NM	β	0	
ZAP 196	O157:H7	γ	****	strong

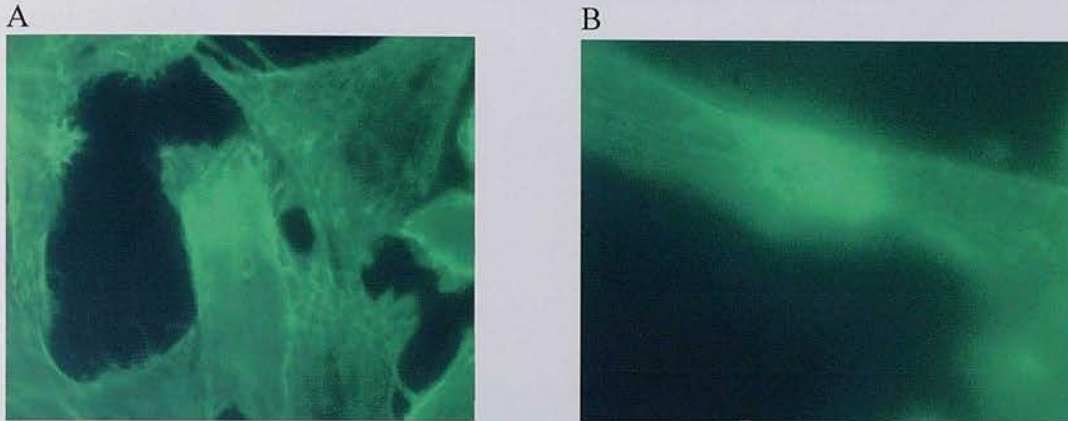
0 – No bacteria expressing intimin, * - <20%, ** - 20 to 40%, *** - 40 to 60%, **** - 60 to 80%, ***** - 80 to 100% estimated proportion of bacteria expressing intimin. Labelling strength estimated by immunofluorescent microscopy.

3.2.14.2 Demonstration of Functional Intimin Expression Using Fluorescent Actin Staining (FAS)

Intimin is a prerequisite for the formation of A/E lesions that can be detected on cultured cells by a method called FAS in which fluorochrome conjugated phalloidin specifically binds to filamentous actin. Regions of fluorescence adjacent to adherent bacteria are indicative of a positive FAS test and so expression of functional intimin can be inferred. Figure 3.14 illustrates the fact that both pICC55 and pSWN01 were capable of expressing functional intimin. All the strains used in this experiment, with the exception of the *eaeA* negative strains, produced a positive FAS test, although lesions produced by the cloned intimin variants were sparse relative to the numbers of adherent bacteria. One explanation for this is that the original *eaeA* clone incorporated a region of 400 base pairs upstream from the transcriptional start site on the assumption that a promoter would be present. It is now known however that *eaeA* is transcribed along with *Tir* and *cesT* on the polycistronic operon *LEE5* which is under the control of a promoter upstream of *Tir* (Sanchez-SanMartin et al., 2001). A certain level of intimin expression occurred from these constructs, as originally shown by Donnenberg and Kaper with pCVD438 (Donnenberg and Kaper, 1991) and subsequently by Frankel with pICC55 (Hartland et al., 2000), presumably from a low level of read-through from an alternative promoter on the plasmid. One consequence of this is that any impaired tissue affinity of these strains relative to the wild types could be due to the low level of intimin expression rather than any functional difference.

Figure 3.14 Positive FAS Reaction by ZAP 204 (A) and ZAP 211 (B)

Areas of actin accumulation (intense green halos) beneath sites of bacterial adherence on HEP-2 cells indicating functional intimin expression from the two cloned *eaeA* constructs used in this study.



3.2.14.3 Affinity of Intimin Deletion Mutants and Derivatives Complemented with Cloned Intimin Types for Intestinal Mucosa

This experiment examined the effect of intimin deletion in both *E. coli* O157:H7 85-170 (ZAP 201) and O26:H- (ZAP 21). The affinity of the intimin deleted 85-170 was not enhanced by complementation with either of the cloned intimin plasmids but both adhered better than the wild type strain (fig. 3.15 and table 3.5). Intimin deletion had no significant effect on affinity levels on any tissue type for the O26 strain and neither did complementation with either of the cloned intimin types (fig. 3.16 and table 3.5). It appeared that in both strains the contribution of intimin to the overall affinity was minimal. The affinity assay used may not, as discussed earlier, have been the most appropriate for quantifying intimin-mediated attachment, as other mechanisms may have accounted for the majority of adherent bacteria observed. If this was the case however, it questions the efficacy of intimin as an initial adhesin. The bacteria that are present on the epithelial surfaces are almost certainly reliant upon a colonisation factor (or factors) since the negative control used in an early IVOC experiment, *L. innocua*, was only observed very rarely on the epithelium in

the IVOC system. Such factors may however simply be involved in survival rather than directly mediating adherence.

Figure 3.15 Affinity of ZAP 201 (*E. coli* O157:H7) and Derivatives to Bovine IVOC

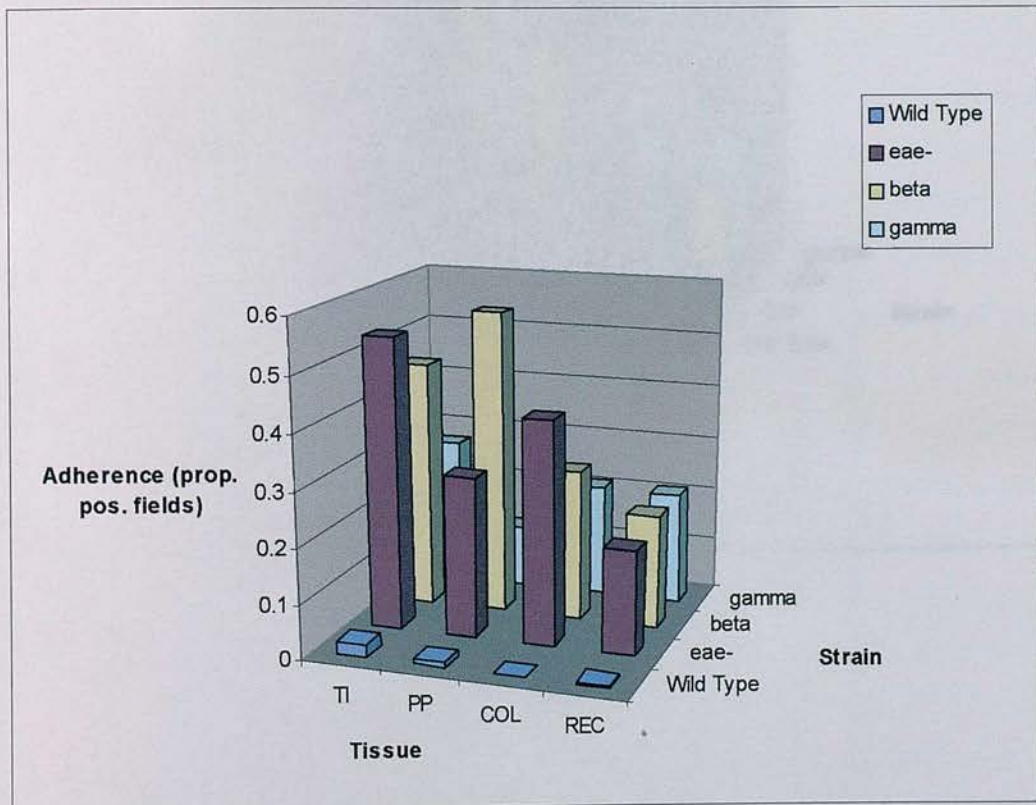


Figure 3.16 Affinity of ZAP 21 and Derivatives (*E. coli* O26:H-) to Bovine IVOC

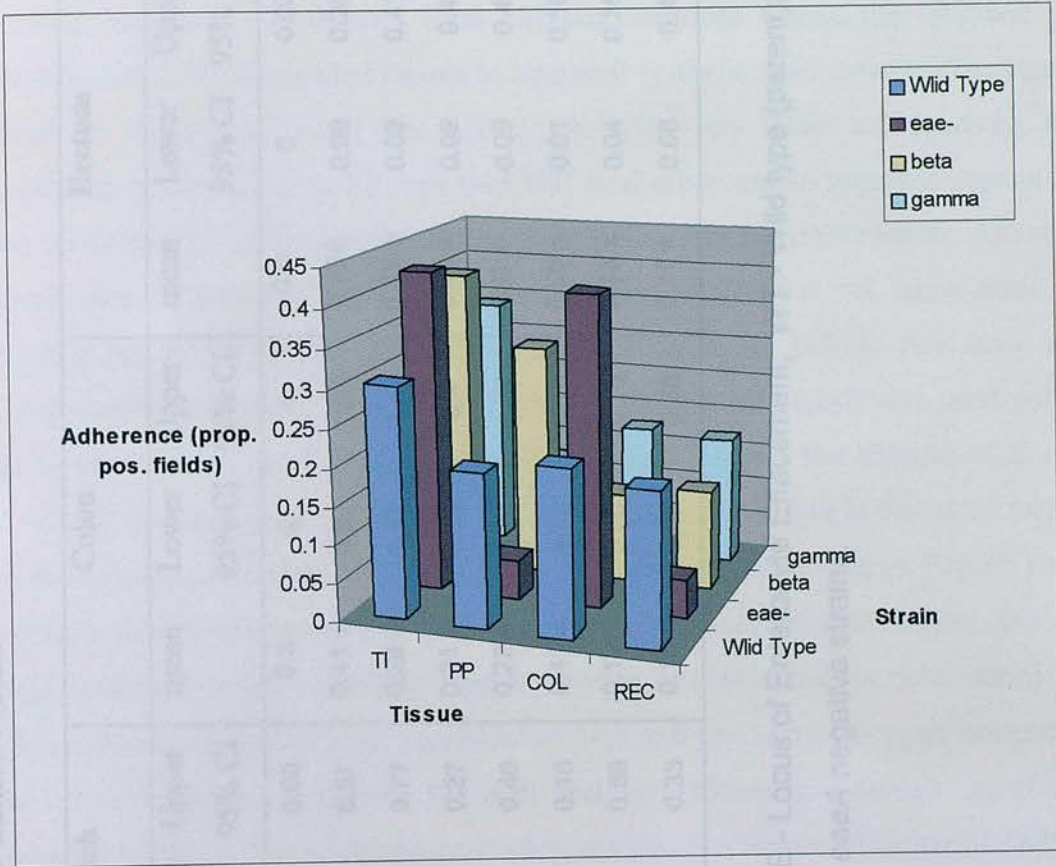


Table 3.3 Affinity of *E. coli* O157:H7 and O26:H- and Derivatives for Bovine Gastrointestinal Epithelia Expressed as the Proportion of Positive Fields

Strain	Verotoxin blood			Peyer's patch		
	Mean	Lower	Upper	Mean	Lower	Upper
ZAP 201	0.62 ± 0.02	0	0.72	0.61 ± 0.01	0	0.69
ZAP 202	0.64 ± 0.01	0.31	0.75	0.29 ± 0.02	0.02	0.57
ZAP 210	0.46 ± 0.05	0.05	0.68	0.26 ± 0.02	0.02	0.47
ZAP 204	0.27 ± 0.01	0.11	0.54	0.11 ± 0.04	0.04	0.27
ZAP 21	0.54 ± 0.03	0.09	0.69	0.2 ± 0.07	0.07	0.46
ZAP 25	0.67 ± 0.01	0.22	0.66	0.03 ± 0.02	0.02	0.16
ZAP 211	0.4 ± 0.01	0.21	0.65	0.31 ± 0.02	0.17	0.69
ZAP 212	0.36 ± 0.01	0.14	0.62	0.02 ± 0.04	0.04	0.33

CI = 95% Confidence Interval; Esp = *E. coli* secreted protein; LEE = Locus of Enterocyte Adhesion; Neg = Negative; N/A indicate intimin type used to complement the *hlyE* negative strain

Table 3.5 Affinity of *E. coli* O157:H7 and O26:H- and Derivatives for Bovine Gastrointestinal Explants Expressed as the Proportion of Positive Fields

Strain	Site	Terminal Ileum			Peyer's patch			Colon			Rectum		
		mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI	mean	Lower 95% CI	Upper 95% CI
ZAP 201	O157 WT	0.02 ±	0	0.12	0.01 ±	0	0.09	0 ±	0	1	0 ±	0	0.03
ZAP202	O157 Neg	0.54 ±	0.31	0.75	0.29 ±	0.12	0.57	0.41 ±	0.21	0.64	0.19 ±	0.08	0.38
ZAP 210	O157 β	0.46 ±	0.25	0.68	0.56 ±	0.33	0.77	0.28 ±	0.13	0.49	0.21 ±	0.09	0.41
ZAP 204	O157 γ	0.27 ±	0.11	0.54	0.11 ±	0.04	0.27	0.21 ±	0.09	0.4	0.21 ±	0.09	0.4
ZAP 21	O26 WT	0.3 ±	0.08	0.69	0.2 ±	0.07	0.46	0.22 ±	0.08	0.47	0.2 ±	0.09	0.4
ZAP 25	O26 Neg	0.42 ±	0.22	0.65	0.05 ±	0.02	0.16	0.41 ±	0.12	0.78	0.05 ±	0.01	0.18
ZAP211	O26 β	0.4 ±	0.21	0.63	0.31 ±	0.12	0.59	0.12 ±	0.05	0.26	0.13 ±	0.04	0.35
ZAP 212	O26 γ	0.34 ±	0.14	0.62	0.12 ±	0.04	0.33	0.18 ±	0.08	0.36	0.17 ±	0.06	0.4

CI - 95% Confidence Interval; Esp - *E. coli* secreted protein; LEE - Locus of Enterocyte Effacement; WT - Wild type (parent); Neg - Negative; β/γ indicate intimin type used to complement the *eaeA* negative strains.

3.3 Summary

The above experiments simultaneously examined two aspects of *E. coli* O157:H7 biology, tropism to epithelium from different locations within the GIT and the contribution of *LEE*-encoded factors to intestinal epithelium adherence. The results must be interpreted taking into account the discovery made subsequently, and described in Chapter 5, that *E. coli* O157:H7 strains possess an apparent tropism for the epithelium of the lymphoid follicle rich mucosa at the terminal rectum. Although rectal mucosa was used in both IVOC experiments this was not taken from the vicinity of the recto-anal junction where the lymphoid follicle rich area was subsequently identified. However a lymphoid follicle rich tissue was used within these experiments, the Peyer's patch of the terminal ileum. The absence of *E. coli* O157:H7 on the Peyer's patch of the persistently shedding calves is discussed further in the discussion sections of Chapter 5. The reduced affinity levels on Peyer's patch of the high secretors relative to the low secretors may have been an indirect result of enhanced LER levels. However work by Roe *et al* (submitted for publication) has shown that LER levels do not vary between high and low secretors, again suggesting that the differences observed for high and low secretors, although significant statistically, were a result of strain variation of unknown factors. The use of ZAP 26 and its isogenic *LEE* negative derivative, ZAP 27, eliminated the uncertainty resulting from strain to strain variation and provided evidence of a novel bovine colonisation factor for *E. coli* O157:H7.

The observations that deleting the *LEE* or intimin did not adversely affect the levels of affinity demonstrated that at least with this methodology, the contribution of intimate attachment to the initial adherence to bovine epithelium was minimal. Intimate attachment is certainly an essential step in the colonisation of cattle as shown by Cornick *et al* (Cornick *et al.*, 2002) but it is unlikely that a *LEE* factor is mediating an initial adherence step on the tissue examined.

4.1 Attributes of a Good *In Vivo* Model of Persistence

In order to investigate the mechanisms of colonization *in vivo*, a model system of persistent *E. coli* O157:H7 carriage must be established. Such a model has the potential to uncover many aspects of *E. coli* O157:H7 biology, both by direct observation, as described in Chapter 5, by growing mutants that lack putative colonization factors and by more sophisticated molecular techniques such as signature-tagged mutagenesis (STM) (Hessel et al., 1995) or recombinant based *in vivo* expression technology (RVET) (Canniffe et al., 1994). Any deviation from the natural conditions of colonization may compromise the potential of the model to produce conclusions relevant to the natural situation. The conditions faced by the organism in the natural environment therefore must be mimicked in the model. The term "model" in this context refers to a system in which the infectious agent of interest is introduced by an alternative species, such as the mouse or rabbit, that is more convenient to work with than the natural host. In this study however, we were fortunate enough to be using the natural host of most interest for the organism being studied. This should still be considered as a model since the acquisition of the organism differs from the natural situation in one important respect, the size of the initial inoculum.

Work by T.E. Besser et al. (Besser et al., 2001) has shown that a challenge of 10^9 CFU of the challenge strain to a group of 100 calves it was determined that 10^9 CFU was required for the majority of calves within a group to become colonized. It is difficult to determine the number of bacteria ingested during natural acquisition of the organism, it is therefore difficult to determine the minimum number of bacteria that may one time be ingested by a calf. It is therefore difficult to determine the number of calves that would become colonized in a group of cattle containing one or more shedding individuals, would result in a population becoming persistently colonized.

In *in vivo* models of colonization and persistence represent complex systems with multiple factors contributing to the success or failure of challenging any individual. Such classic systems reflect the strategy of natural colonization, which are their main advantage. In order to overcome the effects of many unknown factors

A Calf Model of Shedding and Persistence of *E. coli* O157:H7

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4.1 Attributes of a Good *In Vivo* Model of Persistence

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In vivo models of colonisation and persistence represent complex systems with multiple factors contributing to the success or failure of challenging any individual. Such chaotic systems reflect the situation of natural colonisation, which are their main advantage. In order to overcome the effects of many unknown factors

groups of challenged animals can be compared directly provided that every factor, other than the one being tested is consistent between the two groups. Variables that should be consistent between the different groups include diet, housing, mean age, the conditions in which they were reared, inoculation size, background strain and the method used to quantify shedding. Even though the shedding level and duration may vary considerably between animals within a group, any difference attributable to the factor being examined can be assessed using statistical analyses such as Student's T-test, ANOVA (analysis of variance) (Wardlaw, 1989) or the GLMM (generalised linear mixed model) (Brown and Prescott, 1999). The magnitude of the effect of the factor being tested and the within group variation determine the number of animals per group required to determine whether any difference is statistically significant.

4.2 Rational for the Design of an Experimental Calf Model of *E. coli* O157:H7 Persistence and Colonisation

Establishing a calf colonisation model provides a highly relevant system in which to test single factors that contribute to the colonisation and persistence. Deletion mutants and their complemented derivatives can be generated by genetic manipulation. The presence of the Stx bacteriophage in the majority of *E. coli* O157:H7 isolates classify them as Hazard Group 3 pathogens and Containment Level 3 animal housing facilities must be used for such experiments. Good experimental design requires that as many individuals as possible are incorporated into the study. Using calves in containment level 3 housing already restricts the numbers and further drop-outs from the study due to an efficient inoculation procedure could prevent any potential observations from being demonstrated statistically. If Stx is not an essential colonisation factor however then Stx negative *E. coli* O157:H7 strains can be used instead within less prohibitive facilities.

The calf colonisation model described in this chapter was designed to reflect as closely as possible the conditions of a natural infection. For many reasons (e.g. cost, ease of handling) it is preferable to use very young animals (less than 1 week) in such experiments and in the case of gnotobiotic calves there is the additional benefit of not having a native commensal microbiota that would potentially be a source of between animal variation. *E. coli* O157:H7 does in fact colonise young

calves successfully and several groups have chosen this age range for their experimental challenge system but for various reasons it may not be the most appropriate. For example *E. coli* O157:H7 causes clinically overt disease in neonatal calves. It is tempting to make use of this age group as a model since the colonisation and host responses are apparently exaggerated. However, several epidemiological papers have observed a peak prevalence of *E. coli* O157:H7 carriage within weaned calves (Nielsen et al., 2002). This may be due to unidentified husbandry factors that enhance the likelihood of exposure to the organism but various host factors may also account for this. For example changes in diet, the commensal microflora and the immune status. In order to include these and any other potentially important host factors, the calf persistence model described in this chapter used calves reared conventionally on the farm of origin until at least 2 weeks post-weaning.

Major dietary change at weaning stimulates development from a monogastric into the ruminant digestive system and the commensal microflora changes radically to take advantage of the different nutrients available. It has been suggested that commensal bacteria interact with intestinal epithelium and are required for normal differentiation (Hooper et al., 2002). Pre-weaned and especially neonatal or gnotobiotic calves may therefore represent a marked deviation from the natural environment of *E. coli* O157:H7. Major dietary changes at any stage of life have a marked effect on the GIT physiology of ruminants (Meylan et al., 2002) due to their reliance upon a stable commensal population that adapts to make optimum use of the nutrients available in the diet. Various studies have shown that dietary changes affect the levels of faecal *E. coli* O157:H7 (Harmon et al., 1999; Kudva et al., 1995) in ruminants and fasting promotes the growth of *E. coli* in general. It is therefore important for animals being used in *E. coli* O157:H7 persistence studies to have adapted to a particular diet and feeding regime prior to challenge, and to maintain this for the duration of the experiment.

Choice of the challenge strain is important when establishing a persistence model. Due to the importance of this organism as a cause of human food-borne intestinal disease, a large number of strains from clinical cases and from farm and abattoir surveys have been stored in collections. These strains probably vary in their ability to persist within any model system and it is possible that only a subset of

human isolates have the potential to persistently colonise calves. Conversely it may be possible that many bovine isolates have limited virulence for humans. Ideally therefore, strains that have been associated with human disease and shown to originate in cattle should be used. To minimise the possibility of genetic mutation and selection during transmission or passage through the human host, the bovine isolate should ideally be used.

4.3 The Contribution of Stx to the Colonisation of *E. coli* O157:H7 in the Bovine Intestinal Tract

4.3.1 Colonisation of ZAP 193 in a Group of Four Weaned Calves

Containment level 3 experiments are restricted to small-scale comparisons that are expensive, time consuming and may not include sufficient numbers of animals to statistically detect subtle differences in colonisation ability. Stx negative *E. coli* O157:H7 isolates are rare however and do not have full human virulence potential. The Stx bacteriophage can be lost spontaneously *in vitro* during multiple passages, however additional factors could simultaneously be lost. An alternative is to use a human isolate that has spontaneously lost the Stx bacteriophage within the host environment. ZAP 193 (NCTC 12900) was isolated from a human patient, although it is not known whether Stx was lost prior to human infection, in which case this isolate may possess additional virulence or colonisation factors or differ from the majority of *E. coli* O157:H7 isolates in other ways. ZAP 193 was used in the first attempt to challenge a group of calves (Calf Challenge Experiment 1) and this only persisted beyond 11 days PI in one individual out of four. It was not clear whether this isolate was genuinely poor at colonising calves, the challenge protocol was flawed or if colonisation by any *E. coli* O157:H7 is genuinely hit or miss. A further possibility was that Stx itself was acting as a colonisation factor within the bovine GIT. This possibility is reinforced by the fact that Stx is present in the majority of *E. coli* O157:H7 bovine isolates and the presence of GB₃ receptors on the surface of epithelial cells in the basal crypts of bovine intestinal mucosa implies a role for Stx in this host. However there is some *in vitro* evidence that this strain is not representative of its serotype. In cell culture infections it adheres to and forms FAS positive microcolonies at a much higher level than other *E. coli* O157:H7 isolates. It

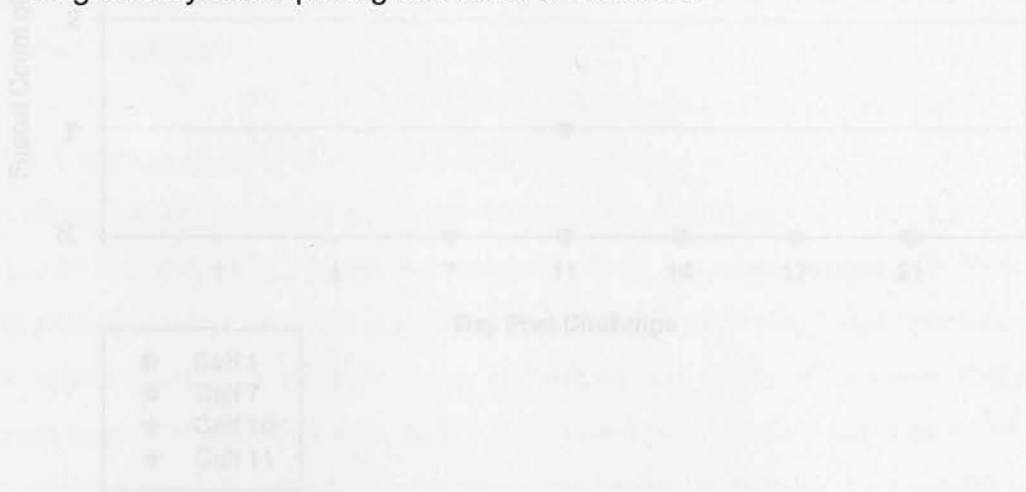
is possible therefore that an enhanced ability to form A/E lesions results in a shorter duration of colonisation.

Table 4.1 Shedding Data from CCE1

Calf	Day Post Challenge						
	1	4	7	11	14	17	21
1	146535	20000	260	N	N	N	N
7	103167	7000	2251	2450	N	N	N
10	440909	800	N	N	N	N	N
11	3417	29000	77500	P	15108	11682	2227

Values indicate most probable number expressed as CFUg⁻¹ of faeces.

N – Negative by direct plating and broth enrichment

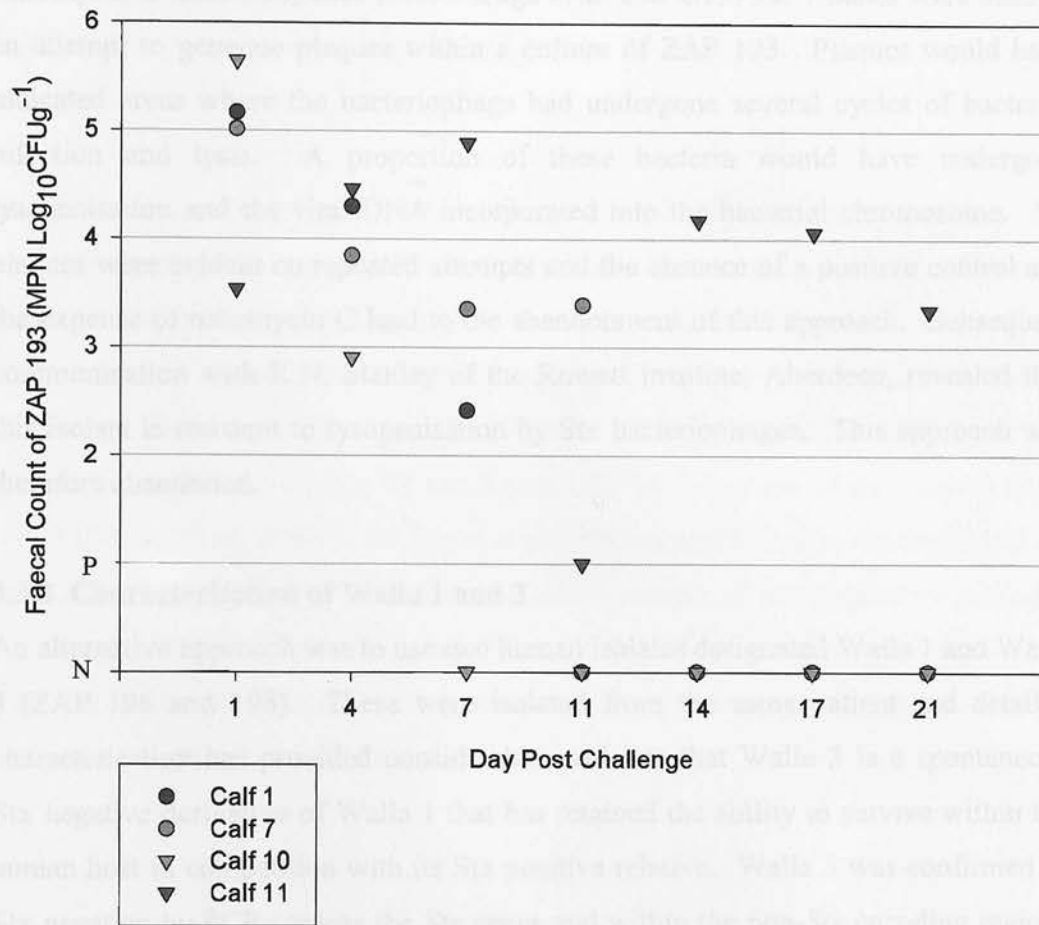


Scatter-graph illustrating the shedding profile of ZAP 193. Each plot represents the Log₁₀ of the most probable numbers (CFUg⁻¹) of nalidixic acid resistant *E. coli* O157:H7 in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.

4.3.2 Typogenetics of ZAP 193 with O157 Six Bacteriophage

One approach to investigate the role of *stx* and also to rule in or out the possible use of Hazard Group 2 strains for cell colonisation experiments, is to create isogenic strains that differ in the presence of a *stx* gene. This had not been performed for any *E. coli* O157:H7 at the time of this study and difficulties arising from the fact that both allelic exchange, an important component of gene deletion, and phage mediated

Figure 4.1 Shedding Data from CCE1



Scatter-graph illustrating the shedding profile of ZAP 193. Each plot represents the Log_{10} of the most probable numbers (CFUg^{-1}) of nalidixic acid resistant *E. coli* O157:H7 in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.

4.3.2 Lysogenisation of ZAP 193 with O157 Stx Bacteriophage

One approach to investigate the role of Stx and also to rule in or out the possible use of Hazard Group 2 strains for calf colonisation experiments, is to create isogenic strains that differ in the presence of a Stx gene. This had not been performed for any *E. coli* O157:H7 at the time of this study and difficulties arising from the fact that both allelic exchange, an important component of gene deletion, and phage mediated

lysis involve RecA recombination were anticipated. The approach chosen was to lysogenise ZAP 193 with a Stx encoding bacteriophage (James et al., 2001). Mitomycin C induced lysates from a range of *E. coli* O157:H7 isolates were used in an attempt to generate plaques within a culture of ZAP 193. Plaques would have indicated areas where the bacteriophage had undergone several cycles of bacterial infection and lysis. A proportion of these bacteria would have undergone lysogenisation and the viral DNA incorporated into the bacterial chromosome. No plaques were evident on repeated attempts and the absence of a positive control and the expense of mitomycin C lead to the abandonment of this approach. Subsequent communication with K.N. Stanley of the Rowett Institute, Aberdeen, revealed that this isolate is resistant to lysogenisation by Stx bacteriophages. This approach was therefore abandoned.

4.3.3 Characterisation of Walla 1 and 3

An alternative approach was to use two human isolates designated Walla 1 and Walla 3 (ZAP 196 and 198). These were isolated from the same patient and detailed characterisation has provided considerable evidence that Walla 3 is a spontaneous Stx negative derivative of Walla 1 that has retained the ability to survive within the human host in competition with its Stx positive relative. Walla 3 was confirmed as Stx negative by PCRs across the *Stx* genes and within the non-*Stx* encoding regions of the Stx-bacteriophage, confirming that the bacteriophage had been lost from the chromosome. A reverse passive latex agglutination based Stx detection kit confirmed that Walla 3 did not release either Stx 1 or Stx 2 following a polymyxin B extraction procedure.

In order to further validate the claim that the two Walla strains were closely related and could therefore be considered isogenic, several established typing methods were utilised. PCR of several known and putative *E. coli* O157:H7 virulence factors confirmed their presence (*eaeA*, *toxB*, *EspP*, *EspA*, *EspB*, *EspD* and *Lif*). They were both phage type 32 as determined by the Scottish *E. coli* O157:H7 reference laboratory. Pulsed-field gel electrophoresis of *Xba*I chromosomal digests of Walla 1 and 3 were overdigested and therefore untypable. Occasional *E. coli* O157:H7 isolates are untypable by this method, so despite the lack of a banding

pattern to analyse, this procedure provides additional evidence that the Walla strains are closely related. The Walla strains demonstrated identical abilities to express and secrete *LEE* encoded proteins under permissive *in vitro* conditions (table 3.2). *E. coli* O157:H7 strains are highly heterogeneous for this phenotype (McNally et al., 2001).

4.3.4 Comparison of Colonisation Ability of Walla 1, Walla 3 and ZAP 3

A direct comparison between Walla 1 and Walla 3 was made in a calf colonisation model. These strains in addition to ZAP 3 were used to challenge three groups of four calves in the Moredun Research Institute Containment Level 3 facility (Calf Challenge Experiment 2). Both Walla 1 and ZAP 3 colonised at least 3 out of 4 calves beyond 17 days (table 4.2 and figure 4.2). However one of the 4 calves due to be challenged with Walla 3 developed signs of pneumonia prior to challenge and was euthanased on humane grounds and to prevent transfer of any respiratory pathogens to the other calves within the group. Only in one of the remaining calves however was the challenge strain detected in the faeces at any point post-challenge and in this individual it persisted to 21 days PI.

Table 4.2 Shedding Data from CCE2

Strain	Calf	Day Post Challenge						
		3	7	10	14	17	21	24
Walla 3	81	N	N	N	N	N	N	N
	106	N	N	N	N	N	N	N
	146	33	25950	58800	8450	150	P	N
Walla 1	87	P	2950	6950	364500	17772	300	2200
	95	5500	2750	222000	244500	9450	P	N
	117	N	P	P	N	N	N	N
	111	4500	800	P	4200	3900	200	P
ZAP 3	118	ND	100	P	N	N	700	1250
	108	9200	50	12400	P	350	10750	150
	121	43000	38300	19200	457500	320000	229000	ND
	140	1850	2850	5150	4150	150	150	P

Values indicate most probable number expressed as CFUg⁻¹ of faeces.

N – Negative by direct plating and broth enrichment

P – Positive by broth enrichment but not detected by direct plating

ND – Not Determined

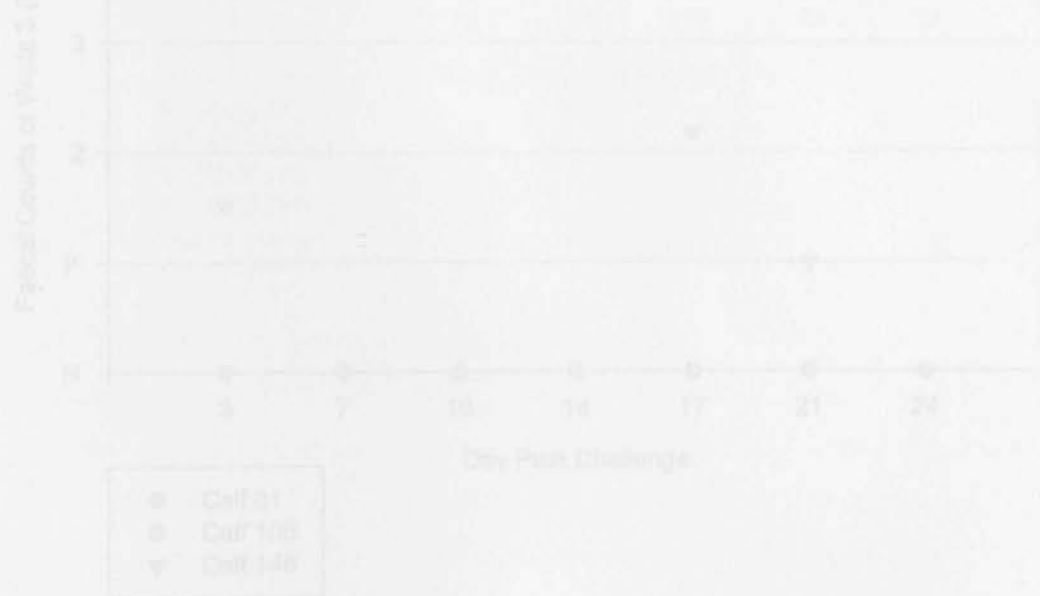
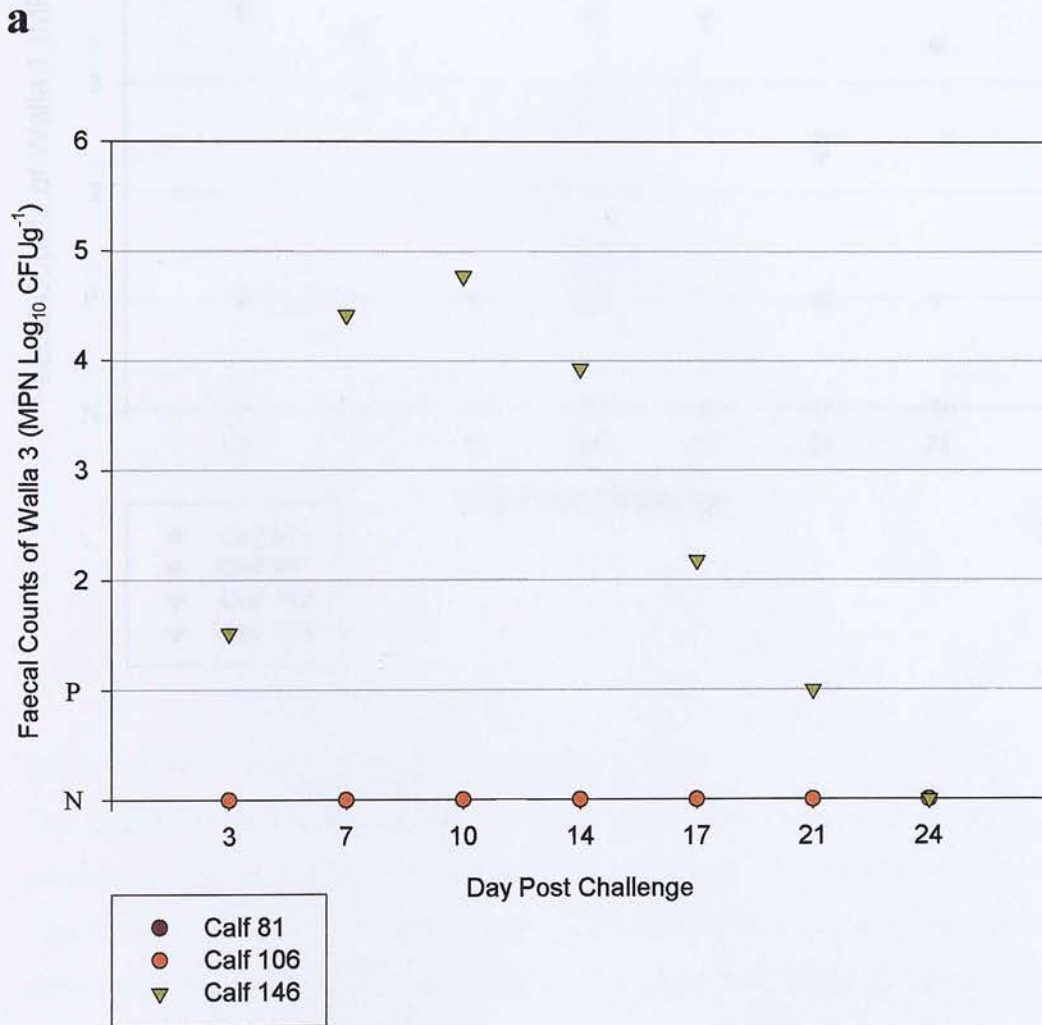
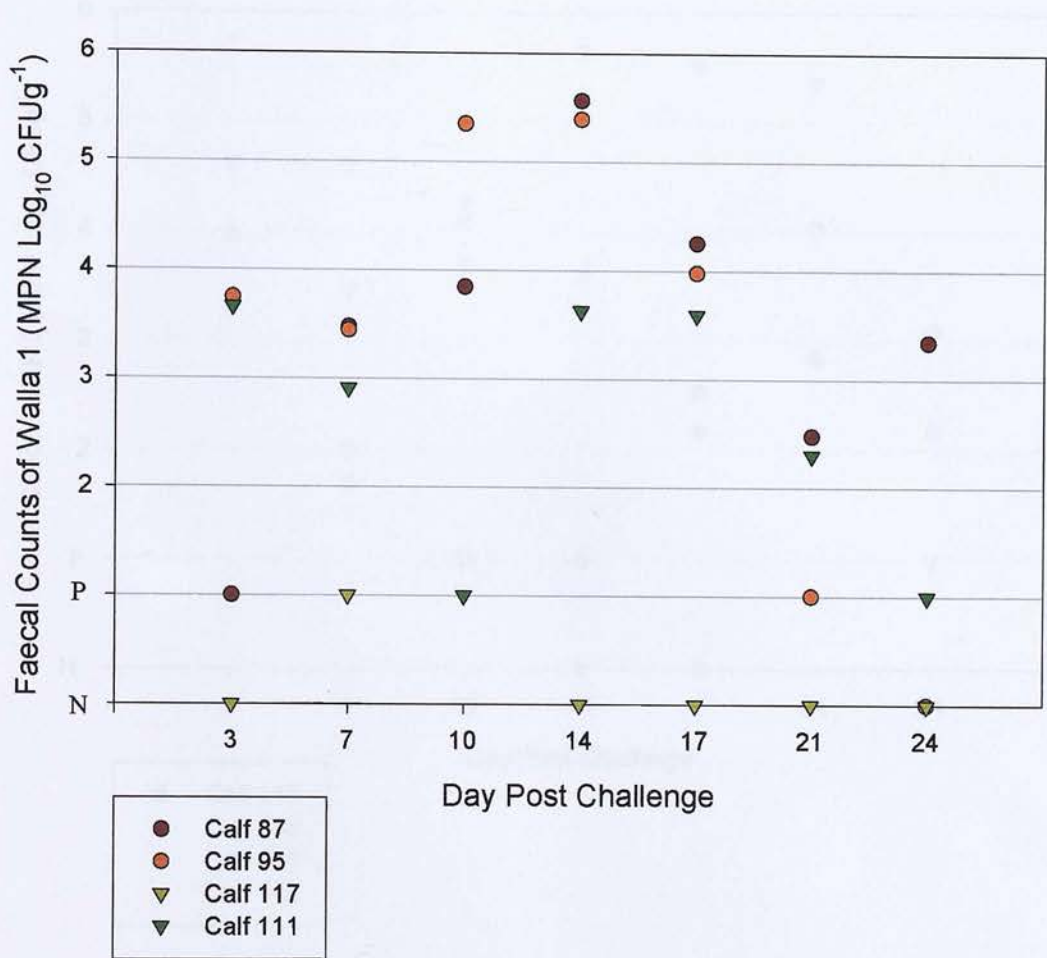


Figure 4.2 Shedding Data from CCE2.

Scatter-graphs illustrating the shedding profile of Walla 3 (a), Walla 1 (b) and ZAP 3 (c). Each plot represents the Log_{10} of the most probable numbers (CFUg^{-1}) of nalidixic acid resistant *E. coli* O157:H7 in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.



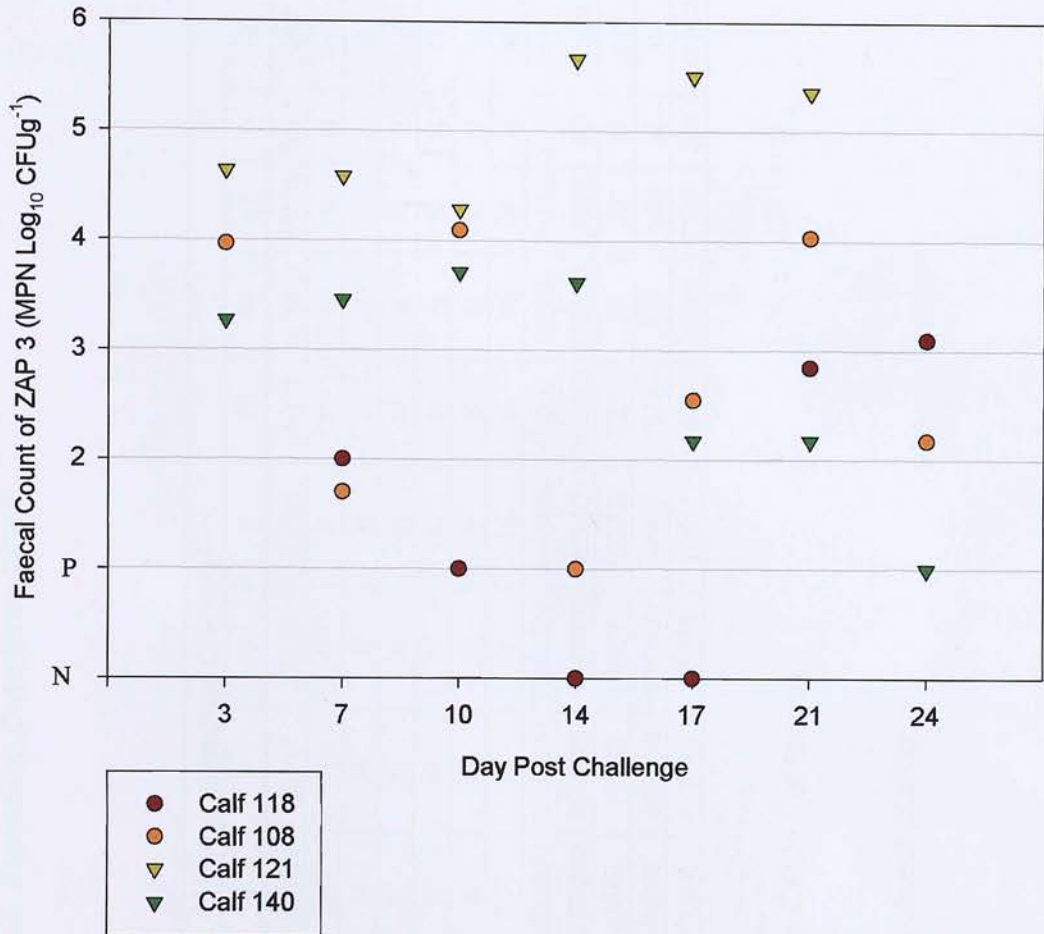
b



4.3.5 Further Comparison of Faecal Counts (2017)

The possibility remained that the two experimental challenges were not equivalent but the number of animals in each group were too small to detect any statistical significance in this regard. The comparison was therefore repeated on a further 6 calves each for both Walla 2 and Walla 1 challenges (Experiment 3). 4 out of six 6 animals challenged with Walla 1 were negative for 24 days or more post challenge. Unexpectedly however, only one out of three calves challenged with Walla 2 became colonised and there was no evidence of any challenge trials at any time point in the remaining 3 animals (Table 3.10) (Fig. 4.12).

C



4.3.5 Further Comparison of Walla Strains (CCE3)

The possibility remained that Stx was mediating a colonisation advantage but the numbers of animals in these groups were too small to attribute any statistical significance to this result. This comparison was therefore repeated on a further 6 calves each for both Walla 1 and 3 (Calf Challenge Experiment 3). 4 out of the 6 animals challenged with Walla 3 were colonised for 24 days or more post challenge. Unexpectedly however, only one calf in the group challenged with Walla 1 became colonised and there was no isolation of the challenge strain at any time point in the remaining 5 animals (Table 4.3 and Fig. 4.3).

Table 4.3 Shedding Data from CCE3

Strain	Calif	Day Post Challenge															
		1	2	3	4	5	6	7	8	9	10	14	17	21	24	28	
W1	295	180	N	1375	6700	73583	13818	10817	29150	71633	91273	47969	28636	8061	3067	ND	
	299	N	N	N	N	N	N	N	N	N	N	N	N	N	N	ND	
	307	N	N	N	ND	N	N	N	N	N	N	N	N	N	N	ND	
	313	N	N	N	N	N	N	N	N	N	N	N	N	N	100	ND	
	322	N	N	N	N	N	N	N	N	N	N	N	N	N	N	ND	
	324	P	N	N	N	N	N	N	N	N	N	N	N	N	N	N	ND
W3	296	42658	1396	2242	N	500	217	N	N	N	N	N	N	N	N	N	
	303	6622	1036	32424	140667	921667	3000000	6325000	2240000	9013636	40090	29364	75455	4775	50	N	
	310	57703	15676	142667	365000	ND	2255000	600909	5730000	81982	267273	380333	57606	90	16583	379333	
	312	25360	3739	45697	47182	272333	40667	19152	2909	2909	727	N	33	N	67	1500	
	315	13063	856	1030	39303	180333	48292	632333	210909	4505	1515	121	100	N	N	N	
	323	7227	3018	12152	17545	33303	77606	176667	88333	18417	9273	28242	7727	394	174767	328000	

Values indicate most probable number expressed as CFUg⁻¹ of faeces.

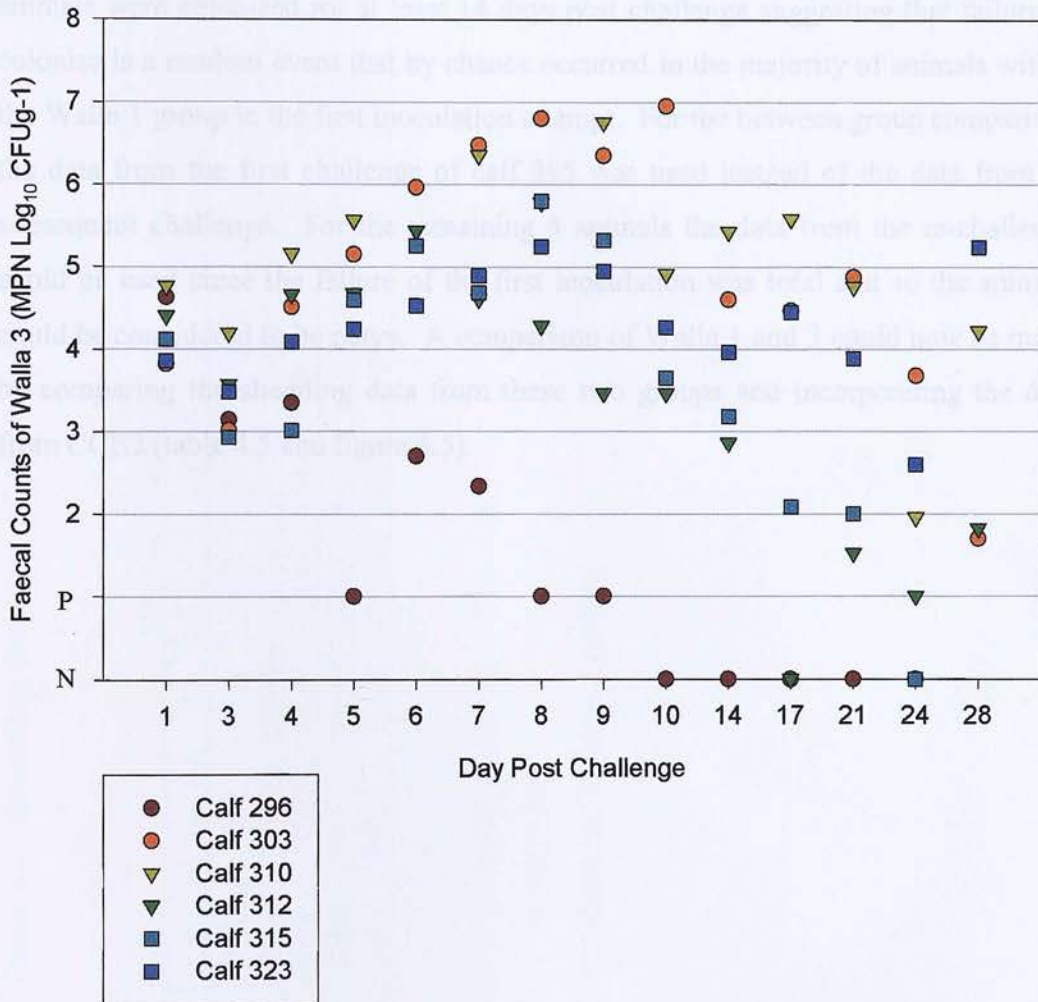
N – Negative by direct plating and broth enrichment

P – Positive by broth enrichment but not detected by direct plating

ND – Not Determined

Figure 4.3 Shedding Data from CCE3 (Walla 3)

Scatter-graph illustrating the shedding profile of Walla 3. Each plot represents the Log_{10} of the most probable numbers (CFUg^{-1}) of nalidixic acid resistant *E. coli* O157:H7 in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.



The phenomenon observed in the Walla 1 group was reminiscent of that in the Walla 3 group in CCE 2, i.e. failure of the initial inoculum to survive the first day and subsequent failure to colonise for the remainder of the observation period in the majority of individuals. The fact that this phenomenon occurred in the group challenged with the Stx positive group suggested that for Walla 3 in CCE 2 the poor

colonisation was not due to the absence of Stx. It was possible that certain individuals were simply refractory to *E. coli* O157:H7 colonisation and that a group can consist mainly of such individuals at certain times simply by chance. An alternative possibility is that certain cultures are in some way deficient at surviving *in vivo*, despite originating from a sweep of colonies rather than an individual colony. To explore these possibilities, the Walla 1 group were all re-challenged with a fresh culture 24 days following the initial inoculation (CCE3b). On this occasion all 6 animals were colonised for at least 14 days post challenge suggesting that failure to colonise is a random event that by chance occurred in the majority of animals within the Walla 1 group in the first inoculation attempt. For the between group comparison the data from the first challenge of calf 295 was used instead of the data from its subsequent challenge. For the remaining 5 animals the data from the re-challenge could be used since the failure of the first inoculation was total and so the animals could be considered to be naïve. A comparison of Walla 1 and 3 could now be made by comparing the shedding data from these two groups and incorporating the data from CCE2 (table 4.5 and figure 4.5).

Table 4.4 Shedding Data from CCE3b (Walla 1)

Strain	Calf	Day Post Challenge													
		1	2	3	4	5	6	7	8	9	10	14	17	21	24
Walla 1	295	18739	145000	145000	24567	4848	18933	13429	41512	22024	4581	9095	P	75717	61286
	299	P	4767	45156	298500	1025000	1960000	122500	1368293	697561	407391	305833	260455	247667	275333
	307	811	91	424	3903	11909	14250	4000	150000	151371	32439	36366	ND	ND	ND
	313	631	3939	80129	70968	304000	803000	996667	647619	1200000	1373333	294091	15315	212	P
	324	225	1043	5000	16364	60226	15531	224750	38605	9000	2130	515	214	17	N

Values indicate most probable number expressed as CFUg⁻¹ of faeces.

N – Negative by direct plating and broth enrichment

P – Positive by broth enrichment but not detected by direct plating

ND – Not Determined

Figure 4.4 Shedding Data from CCE3b (Walla 1)

Scatter-graph illustrating the shedding profile of Walla 1. Each plot represents the Log_{10} of the most probable numbers (CFUg^{-1}) of nalidixic acid resistant *E. coli* O157:H7 in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.

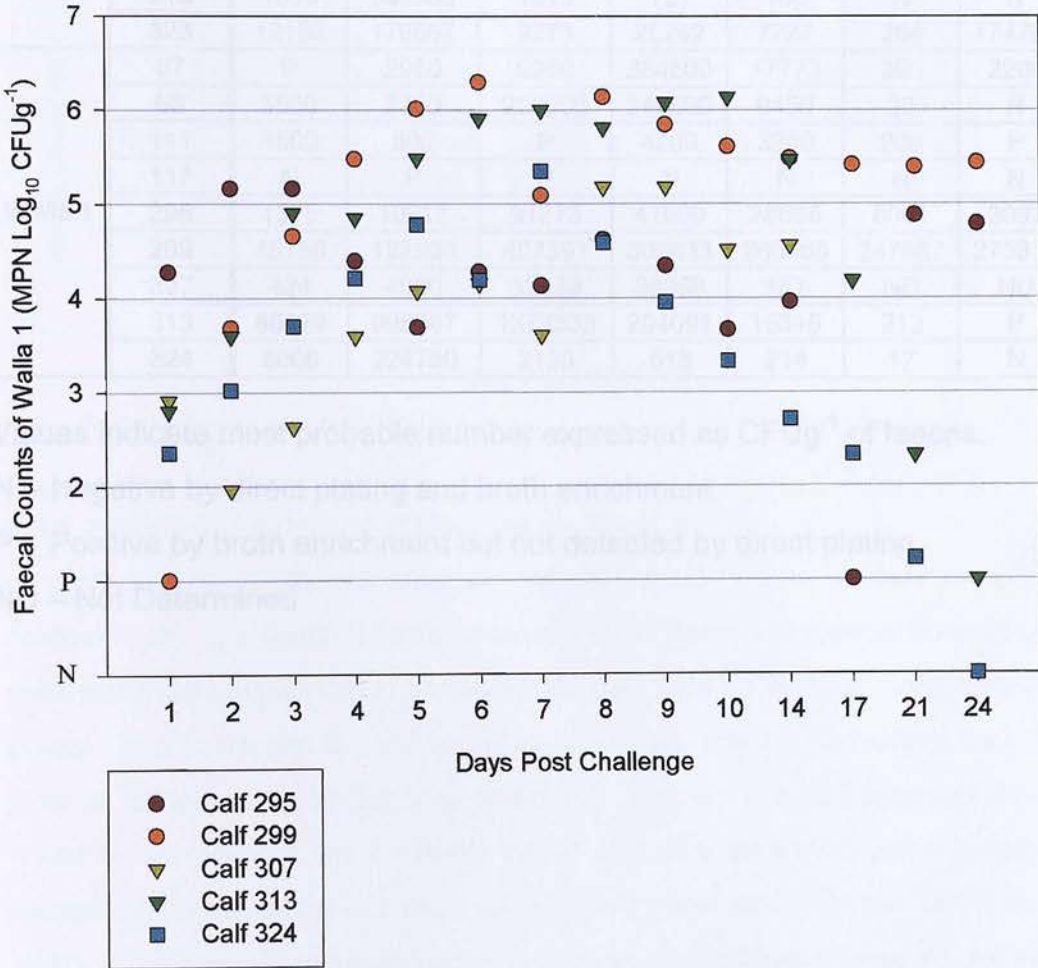


Table 4.5 Comparison of Walla 1 and Walla 3 (all results)

Strain	Calf	Day Post Challenge						
		3	7	10	14	17	21	24
Walla 3	146	P	25950	58800	8450	150	P	N
	296	2242	P	N	N	N	N	N
	303	32424	6325000	40090	29364	75455	4775	50
	310	142667	600909	267273	380333	57606	90	16583
	312	45697	19152	727	N	33	P	67
	315	1030	632333	1515	121	100	N	N
	323	12152	176667	9273	28242	7727	394	174767
Walla 1	87	P	2950	6950	364500	17773	300	2200
	95	5500	2750	222000	244500	9450	30	N
	111	4500	800	P	4200	3900	200	P
	117	N	P	P	N	N	N	N
	295	1375	10817	91273	47969	28636	8061	3067
	299	45156	122500	407391	305833	260455	247667	275333
	307	424	4000	32439	36366	ND	ND	ND
	313	80129	996667	1373333	294091	15315	212	P
324	5000	224750	2130	515	214	17	N	

Values indicate most probable number expressed as CFUg⁻¹ of faeces.

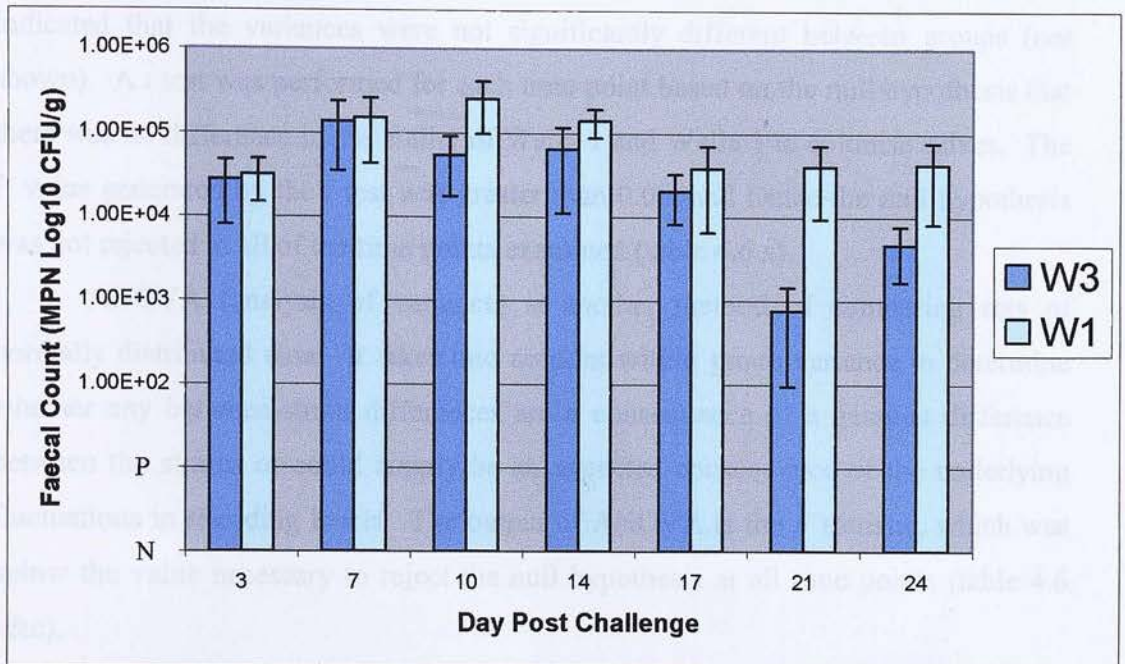
N – Negative by direct plating and broth enrichment

P – Positive by broth enrichment but not detected by direct plating

ND – Not Determined

Figure 4.5 Comparison of Walla 1 and Walla 3 (all results)

Comparison of the mean shedding levels of Walla 1 and Walla 3 over all animals challenged at each time point. Error bars indicate standard error.



4.3.6 Statistical Analysis of Walla 1 versus Walla 3

In order to compare the shedding data of the animals within the two groups and determine any significant differences between the strains, one approach would be to use a statistical analysis that encompasses the data from all the time points from each animal. Due to the fact that the faecal bacterial concentration recorded at each time point is influenced by earlier time points, i.e. they are repeated measures data, it would be necessary to use a suitable model such as a covariance pattern model, a random coefficient model or a more sophisticated mixed model (Brown and Prescott, 1999). Such analysis requires specialist skills and sophisticated computer software. Initially therefore the Walla 1 and Walla 3 shedding data from each time point were compared separately to determine which if any, exhibited significant differences between the groups. More sophisticated techniques could be applied if there were any interesting or inconclusive results.

A simple method of determining significant differences between two sets of data is the Student's *t* test. This analysis requires that the data is normally distributed, and that the two groups possess homogeneous variance, which can be

determined using the rankit procedure and Bartlett's test respectively (Wardlaw, 1989). In order to normalise the data the rankit procedure revealed that a Log_{10} transformation was necessary (not shown). Bartlett's test on the transformed data indicated that the variances were not significantly different between groups (not shown). A t test was performed for each time point based on the null hypothesis that there was no difference in the ability of Walla 1 and Walla 3 to colonise calves. The P value generated by the t test was greater than 0.05, and hence the null hypothesis was not rejected at all of the time points examined (table 4.6 a).

ANOVA (analysis of variance) is another method of comparing sets of normally distributed data. It takes into account within group variance to determine whether any between-strain differences are a consequence of a genuine difference between the strains or could simply be an expected consequence of the underlying fluctuations in shedding levels. The output of ANOVA is the F statistic, which was below the value necessary to reject the null hypothesis at all time points (table 4.6 b&c).

Table 4.6a. t Test Result for the Walla 1 vs Walla 3 Comparison

Day (d)	Mean (W1)	Mean (W3)	Tstat
3	31940.47	31143.45	0.37
7	159094.73	151025.12	0.12
10	61112.82	54121.16	0.28
14	57428.37	53071.22	0.33
17	203369.43	30803.57	1.1
21	7951.78	6951.59	0.25
24	45376.65	42351.28	0.32

Table 4.6c. ANOVA Results for Walla 1 vs Walla 3 Comparison

Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)	Day (d)
3	7	10	14	17	21	24	28	31	34	37	40	43
14	18	21	24	27	30	33	36	39	42	45	48	51
18	21	24	27	30	33	36	39	42	45	48	51	54
21	24	27	30	33	36	39	42	45	48	51	54	57
24	27	30	33	36	39	42	45	48	51	54	57	60
27	30	33	36	39	42	45	48	51	54	57	60	63
30	33	36	39	42	45	48	51	54	57	60	63	66
33	36	39	42	45	48	51	54	57	60	63	66	69
36	39	42	45	48	51	54	57	60	63	66	69	72
39	42	45	48	51	54	57	60	63	66	69	72	75
42	45	48	51	54	57	60	63	66	69	72	75	78
45	48	51	54	57	60	63	66	69	72	75	78	81
48	51	54	57	60	63	66	69	72	75	78	81	84
51	54	57	60	63	66	69	72	75	78	81	84	87
54	57	60	63	66	69	72	75	78	81	84	87	90
57	60	63	66	69	72	75	78	81	84	87	90	93
60	63	66	69	72	75	78	81	84	87	90	93	96
63	66	69	72	75	78	81	84	87	90	93	96	99
66	69	72	75	78	81	84	87	90	93	96	99	102
69	72	75	78	81	84	87	90	93	96	99	102	105
72	75	78	81	84	87	90	93	96	99	102	105	108
75	78	81	84	87	90	93	96	99	102	105	108	111
78	81	84	87	90	93	96	99	102	105	108	111	114
81	84	87	90	93	96	99	102	105	108	111	114	117
84	87	90	93	96	99	102	105	108	111	114	117	120
87	90	93	96	99	102	105	108	111	114	117	120	123
90	93	96	99	102	105	108	111	114	117	120	123	126
93	96	99	102	105	108	111	114	117	120	123	126	129
96	99	102	105	108	111	114	117	120	123	126	129	132
99	102	105	108	111	114	117	120	123	126	129	132	135
102	105	108	111	114	117	120	123	126	129	132	135	138
105	108	111	114	117	120	123	126	129	132	135	138	141
108	111	114	117	120	123	126	129	132	135	138	141	144
111	114	117	120	123	126	129	132	135	138	141	144	147
114	117	120	123	126	129	132	135	138	141	144	147	150
117	120	123	126	129	132	135	138	141	144	147	150	153
120	123	126	129	132	135	138	141	144	147	150	153	156
123	126	129	132	135	138	141	144	147	150	153	156	159
126	129	132	135	138	141	144	147	150	153	156	159	162
129	132	135	138	141	144	147	150	153	156	159	162	165
132	135	138	141	144	147	150	153	156	159	162	165	168
135	138	141	144	147	150	153	156	159	162	165	168	171
138	141	144	147	150	153	156	159	162	165	168	171	174
141	144	147	150	153	156	159	162	165	168	171	174	177
144	147	150	153	156	159	162	165	168	171	174	177	180
147	150	153	156	159	162	165	168	171	174	177	180	183
150	153	156	159	162	165	168	171	174	177	180	183	186
153	156	159	162	165	168	171	174	177	180	183	186	189
156	159	162	165	168	171	174	177	180	183	186	189	192
159	162	165	168	171	174	177	180	183	186	189	192	195
162	165	168	171	174	177	180	183	186	189	192	195	198
165	168	171	174	177	180	183	186	189	192	195	198	201
168	171	174	177	180	183	186	189	192	195	198	201	204
171	174	177	180	183	186	189	192	195	198	201	204	207
174	177	180	183	186	189	192	195	198	201	204	207	210
177	180	183	186	189	192	195	198	201	204	207	210	213
180	183	186	189	192	195	198	201	204	207	210	213	216
183	186	189	192	195	198	201	204	207	210	213	216	219
186	189	192	195	198	201	204	207	210	213	216	219	222
189	192	195	198	201	204	207	210	213	216	219	222	225
192	195	198	201	204	207	210	213	216	219	222	225	228
195	198	201	204	207	210	213	216	219	222	225	228	231
198	201	204	207	210	213	216	219	222	225	228	231	234
201	204	207	210	213	216	219	222	225	228	231	234	237
204	207	210	213	216	219	222	225	228	231	234	237	240
207	210	213	216	219	222	225	228	231	234	237	240	243
210	213	216	219	222	225	228	231	234	237	240	243	246
213	216	219	222	225	228	231	234	237	240	243	246	249
216	219	222	225	228	231	234	237	240	243	246	249	252
219	222	225	228	231	234	237	240	243	246	249	252	255
222	225	228	231	234	237	240	243	246	249	252	255	258
225	228	231	234	237	240	243	246	249	252	255	258	261
228	231	234	237	240	243	246	249	252	255	258	261	264
231	234	237	240	243	246	249	252	255	258	261	264	267
234	237	240	243	246	249	252	255	258	261	264	267	270
237	240	243	246	249	252	255	258	261	264	267	270	273
240	243	246	249	252	255	258	261	264	267	270	273	276
243	246	249	252	255	258	261	264	267	270	273	276	279
246	249	252	255	258	261	264	267	270	273	276	279	282
249	252	255	258	261	264	267	270	273	276	279	282	285
252	255	258	261	264	267	270	273	276	279	282	285	288
255	258	261	264	267	270	273	276	279	282	285	288	291
258	261	264	267	270	273	276	279	282	285	288	291	294
261	264	267	270	273	276	279	282	285	288	291	294	297
264	267	270	273	276	279	282	285	288	291	294	297	300
267	270	273	276	279	282	285	288	291	294	297	300	303
270	273	276	279	282	285	288	291	294	297	300	303	306
273	276	279	282	285	288	291	294	297	300	303	306	309
276	279	282	285	288	291	294	297	300	303	306	309	312
279	282	285	288	291	294	297	300	303	306	309	312	315
282	285	288	291	294	297	300	303	306	309	312	315	318
285	288	291	294	297	300	303	306	309	312	315	318	321
288	291	294	297	300	303	306	309	312	315	318	321	324
291	294	297	300	303	306	309						

Table 4.6a t Test Result for the Walla 1 vs Walla 3 Comparison

Day PC	Mean W3	Mean W1	Ttest
3	27580.47	31748.95	0.87
7	136094.73	151986.14	0.92
10	54112.52	254320.16	0.29
14	64084.37	139912.39	0.33
17	20346.43	38393.57	0.62
21	765.74	40519.56	0.25
24	4679.65	42360.26	0.32

Table 4.6b Tabulated Values of F Statistic

Tabulated Values of F		
P	1/F	F
0.05	245	4.6
0.01	6140	8.86

Table 4.6c ANOVA Result for the Walla 1 vs Walla 3 Comparison

Day PC	Total Degrees of Freedom	Between Group Degrees of Freedom	Within Group Degrees of Freedom	Between Group Sum of Squares	Within Group Sum of Squares	Between Group Variance	Within Group Variance	F	1/F	Day PC
3	15	1	14	0.2499	31.2652	0.2499	2.2332	0.1119	8.9344	3
7	15	1	14	0.0699	36.5974	0.0699	2.6141	0.0267	37.3698	7
10	15	1	14	0.6459	42.7781	0.6459	3.0555	0.2113	4.7305	10
14	15	1	14	5.2093	57.4583	5.2093	4.1041	1.2692	NA	14
17	15	1	14	0.0276	49.8788	0.0276	3.5627	0.0077	128.8243	17
21	15	1	14	1.6923	38.8484	1.6923	2.7748	0.6098	1.6397	21
24	15	1	14	0.0717	59.0131	0.0717	4.2152	0.0170	58.7411	24

4.4 Colonisation of Calves with *E. coli* O26:H-

Shiga-toxigenic *E. coli* O26 strains are frequently associated with the same range of human diseases as *E. coli* O157:H7, i.e. haemorrhagic colitis and haemolytic uraemic syndrome (Zhang et al., 2000). They are also particularly prevalent within the bovine population and in contrast to *E. coli* O157:H7, have been associated with naturally occurring disease in this species (Pearson et al., 1999; Gunning et al., 2001). An *E. coli* O26:H- possessing Stx-1 and isolated from a calf with diarrhoea (Ebel et al., 1996; Wieler et al., 1996) was used to challenge a group of 6 calves, using the model established for *E. coli* O157:H7, to observe not only shedding levels but also any evidence of pathology. Assuming it was the cause of diarrhoea in the calf it was isolated from, the occurrence of similar disease in the challenged calves would have fulfilled Koch's postulates. Examination of the role of a wide range of factors would have been subsequently made possible as the collaborating scientist who provided this strain (Trinad Chakraborty, Giessen, Germany) has produced a number of deletion mutants in the same strain.

Rectal temperatures were recorded daily but remained normal in all calves except one calf when a slight increase in temperature was observed for 3 consecutive days which coincided with production of slightly loose, mucoid faeces. Compared to the *E. coli* O157:H7 strains tested in this model, this strain did not persist for a prolonged period. The inoculum survived in all calves initially, with levels ranging from 5×10^4 to 6.5×10^5 CFU/g, but these levels dropped rapidly and by day 7 PI only one calf was still shedding (table 4.7 and figure 4.7). This animal was enrichment negative by day 17. This pattern was distinct from the majority of "failed" challenges with *E. coli* O157:H7 that typically did not shed the challenge strain in large numbers at any point following inoculation. A notable exception was ZAP 193 that, like O26:H-, initially colonised all animals in the group quite effectively but did not persist in 3 out of the 4 animals beyond day 11. The difference between the O157:H7 strains and the O26:H- strain is illustrated in fig. 4.8 which shows the mean shedding levels across all animals challenged with each strain. On this graph ZAP 193 appears to behave more like the other *E. coli* O157:H7 due to the one animal shedding *E. coli* O157:H7 beyond day 11 shedding fairly high numbers that skews

skews the mean level. In reality the majority of animals in this group behaved like the O26:H- group.

Figure 4.6 Shedding Data from CCE4, O26:H- (ZAP 21)

Scatter-graph illustrating the shedding profile of ZAP 21. Each plot represents the Log_{10} of the most probable numbers (CFUg^{-1}) of nalidixic acid resistant *E. coli* O26:H- in faecal samples. Samples yielding no colonies by direct plating have been labelled P if enrichment positive and N if enrichment negative to facilitate graphical representation.

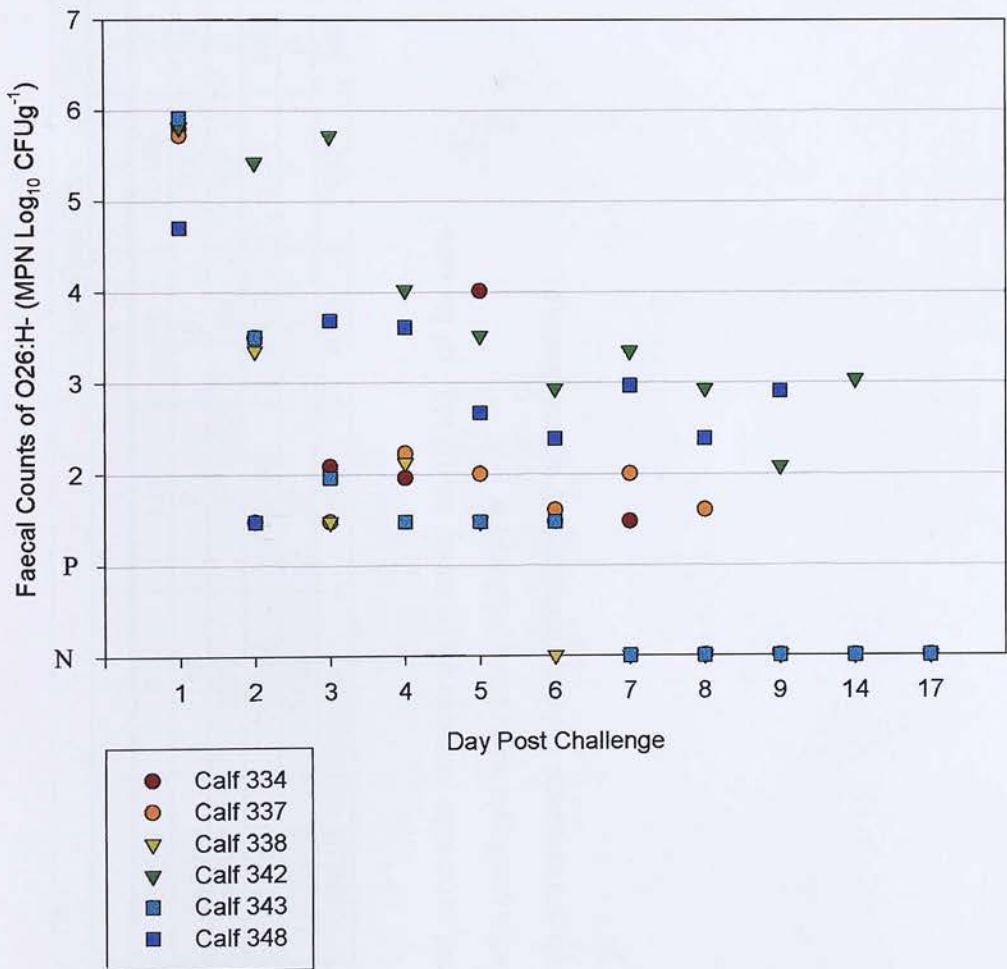


Table 4.7 Shedding Data from CCE4, O26:H- (ZAP 21)

Strain	Calf	Day Post Challenge														
		1	2	3	4	5	6	7	8	9	14	17				
O26:H-	334	632667	P	121	91	10180	P	P	N	N	N	N	N	N		
O26:H-	337	525333	3153	P	167	100	40	100	40	N	N	N	N	N		
O26:H-	338	645667	2252	P	133	30	N	N	N	N	N	N	N	N		
O26:H-	342	658000	265574	520909	10455	3261	850	2188	850	120	1067	N	N	N		
O26:H-	343	819333	3153	91	P	P	P	N	N	N	N	N	N	N		
O26:H-	348	50870	30	4826	4061	462	240	920	240	800	N	N	N	N		

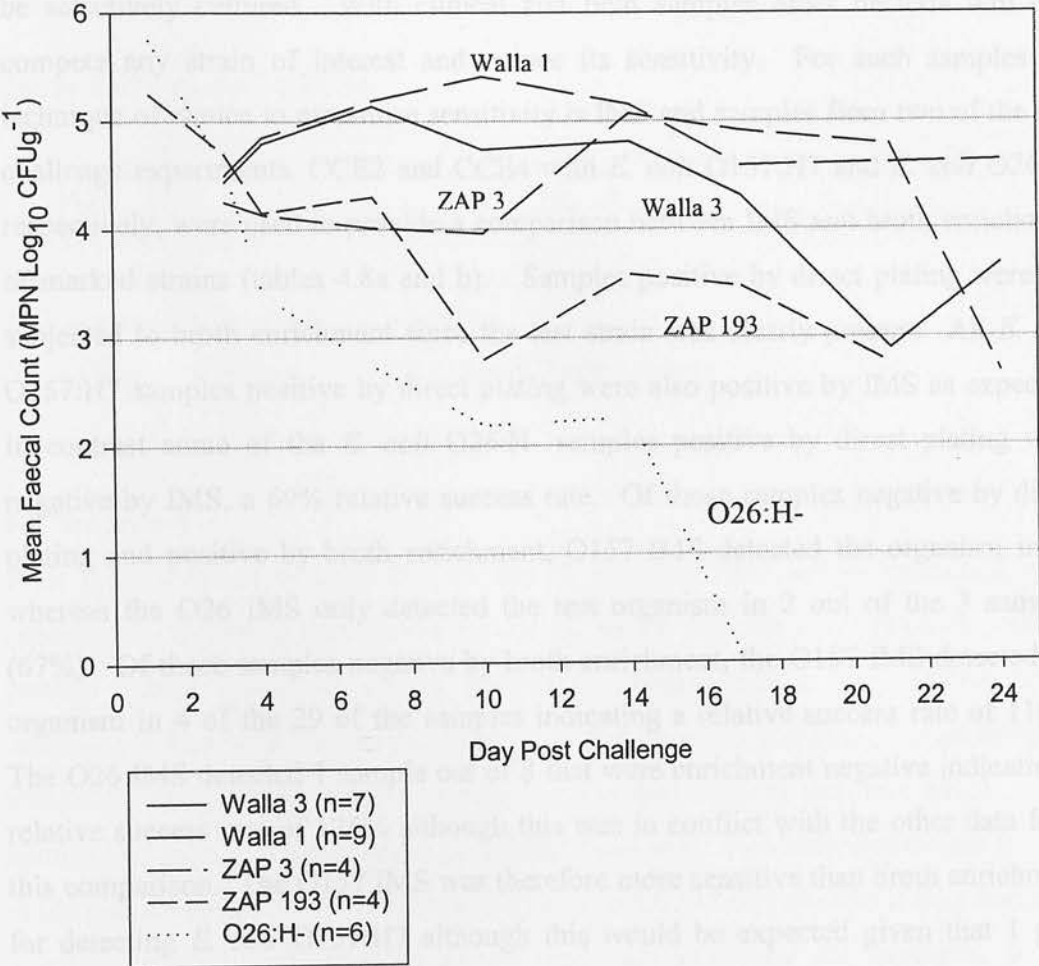
Values indicate most probable number expressed as CFUg⁻¹ of faeces.

N – Negative by direct plating and broth enrichment

P – Positive by broth enrichment but not detected by direct plating

Figure 4.7 Mean Shedding Levels for Each Strain Tested in the Calf Challenge Model

Mean levels over all calves challenged with each strain expressed as \log_{10} most probable number (CFUg^{-1}).



4.5 Comparison of Broth Enrichment and Immunomagnetic Separation (IMS) for Detection of Nalidixic Acid Marked Strains

The estimation of bacterial concentration by direct plating of serial faecal dilutions onto selective media has a limit of sensitivity determined by the quantity of faeces that can be plated out. Increased sensitivity of strains marked by antimicrobial resistance can be achieved by adding samples to a broth and any marked bacteria will be selectively cultured. With clinical and field samples other bacteria will out-compete any strain of interest and reduce its sensitivity. For such samples the technique of choice to maximise sensitivity is IMS and samples from two of the calf challenge experiments, CCE2 and CCE4 with *E. coli* O157:H7 and *E. coli* O26:H- respectively, were used to provide a comparison between IMS and broth enrichment of marked strains (tables 4.8a and b). Samples positive by direct plating were not subjected to broth enrichment since the test strain was clearly present. All *E. coli* O157:H7 samples positive by direct plating were also positive by IMS as expected. In contrast some of the *E. coli* O26:H- samples positive by direct plating were negative by IMS, a 69% relative success rate. Of those samples negative by direct plating and positive by broth enrichment, O157 IMS detected the organism in all whereas the O26 IMS only detected the test organism in 2 out of the 3 samples (67%). Of those samples negative by broth enrichment, the O157 IMS detected the organism in 4 of the 29 of the samples indicating a relative success rate of 116%. The O26 IMS detected 1 sample out of 8 that were enrichment negative indicating a relative success rate of 125% although this was in conflict with the other data from this comparison. The O157 IMS was therefore more sensitive than broth enrichment for detecting *E. coli* O157:H7 although this would be expected given that 1 g of faeces is used for the test compared with 0.1 g for the broth enrichment. The O26 IMS however did not compare favourably with broth enrichment although it is not clear why. It is possible that cross-reactive bacteria within the samples competed for magnetic beads or some other technical problem existed.

Tables 4.8 Comparison of IMS with Broth Enrichment for Detecting NaI^R *E. coli*.

Table 4.8a *E. coli* O157:H7

	Direct Plating +	DP - Broth Enrichment +	DP - BE -
IMS +	42	13	4
IMS -	0	0	25

Table 4.8b *E. coli* O26:H-

	Direct Plating +	DP - Broth Enrichment +	DP - BE -
IMS+	13	2	1
IMS -	4	1	7

IMS – Immunomagnetic Separation

DP – Direct Plating; BE – Broth Enrichment

4.6 Summary

With the exception of ZAP 193 all the *E. coli* O157:H7 strains examined were capable of colonising and persisting for up to 28 days in conventionally reared, weaned calves using the system described. Importantly a Stx negative strain colonised at least as effectively as its Stx positive co-strain validating its use in future studies in containment level 2 which has the potential to examine much larger numbers of calves. This result suggests that there is no absolute requirement for Stx in the persistent colonisation of calves by *E. coli* O157:H7, however it may still play a subtle role in mediating an evolutionary advantage by for example, down-regulating immune responses to either enhance the duration or level of shedding or to facilitate re-colonisation. Any such subtle effect would not be detected in these model systems due to the small numbers of animals and the large variation in colonisation pattern within each group. A further complication could be the presence of other STEC within the calves being challenged, facilitating colonisation of the Stx negative challenged strains by trans-complementation. Such a factor is impossible to control without the use of SPF animals due to the ubiquity of STEC within the farm environment. Such animals themselves represent a potentially flawed model system

due to the abnormal commensal populations and are also prohibitively expensive for large-scale studies, especially within containment level 3.

There are various explanations for the poor colonisation ability of O26:H-. The O26 serogroup may be diverse and consist of strains adapted for different host species and ages. For example, the challenge strain may have adapted to cause a relatively short-term, intense colonisation of compromised or immunologically naïve young animals which would result in clinically apparent disease. Challenge of younger animals e.g. 1 week old, may have resulted in clinically overt disease. The older calves challenged in this study may have experienced subclinical pathology, transiently and subtly evident in only one individual. The use of an O26 isolate from an older animal may have resulted in more persistent colonisation of the weaned calves, perhaps similar to that observed for *E. coli* O157:H7, since such a strain may have been better adapted to the age of calves used in these experiments. If however the colonisation pattern observed was in fact a typical representation of *E. coli* O26 colonisation, then it has implications for the biology of *E. coli* O157:H7, the duration of colonisation of which appears to be far superior. This enhanced colonisation compared with another EHEC serotype, that is both highly prevalent and potentially a pathogen within cattle, suggests the presence of a factor or factors that mediate this advantage. Such factors would clearly increase the prevalence of *E. coli* O157:H7 and thus increase the risk of transmission to the human population. Identification of these factors would therefore provide targets for future control strategies.

E. coli O157:H7 at the
Terminal Rectum

5.1 Background

One strategy to examine the interaction between *E. coli* O157:H7 and the bovine intestinal epithelium is to determine the distribution of the organism in naturally colonised calves and observe the organism within the GIT of experimentally challenged calves. This approach has the potential to highlight which region or regions, if any, is being colonised by *E. coli* O157:H7 as its primary niche. With this objective in mind, it is important to establish an experimental system that mirrors as closely as possible the conditions of natural colonisation, such as the model of colonisation and persistence described in Chapter 4. Methodology and interpretation of results are also important as the GIT is a very dynamic environment and distribution of bacteria throughout different compartments, such as the rumen, small intestine and caecum, could make it difficult to determine which of these is the site of multiplication. It is highly likely that *E. coli* O157:H7 is capable of multiplying in the majority of sites in the bovine GIT to varying extents. Analysis of relative bacterial numbers over a number of animals may highlight a particular region but a widespread distribution would make any such conclusion difficult to reach. However, as will be described in this Chapter, sampling of long-term persistent shedders from an appropriate model system has resulted in the identification of a site of colonisation that not only accounts for the majority of faecal excretion but also has the potential to be a site of multiplication.

Chapter 5

Localisation of

E. coli O157:H7 at the

Terminal Rectum

The experimental challenge of weaned calves with *E. coli* O157:H7 involves natural colonisation and shedding of the organism. The distribution of the organism within the GIT of naturally colonised calves is not well understood. However, *E. coli* O157:H7 has been recovered from all sites sampled within the GIT, except the abomasum, with the highest recovery rate in the first 100 cm. Gray et al (1997) (1993) and Gray et al (1997) reported high numbers in caecum and terminal ileum in large intestinal sites. These studies suggest a variable distribution with no specific tropism for any site within the bovine GIT. However Gray et al (1997) used rumen and duodenal cannulae to sample from live calves and beyond day 10 no *E. coli* O157:H7 was recovered from any of these locations. This was despite a being present in rumen of the corresponding feed samples up to day 36, implying a large intestinal location. None of these studies demonstrated any evidence of mucosal localisation or A/B lesions in persistently shedding animals.

5.1 Background

One strategy to examine the interaction between *E. coli* O157:H7 and the bovine intestinal epithelium is to determine the distribution of the organism at necropsy. Quantification and observation of the organism within the GIT of experimentally challenged calves has the potential to highlight which region or mucosal type, if any, is being utilised by *E. coli* O157:H7 as its primary niche. With this objective it is important to establish an experimental system that mirrors as closely as possible the conditions of natural colonisation, such as the model of colonisation and persistence described in Chapter 4. Methodology and interpretation of results are also important as the GIT is a very dynamic environment and distribution of bacteria throughout different compartments, e.g. epithelial surface, mucus layer and digesta, could make it difficult to determine which of these is the site of multiplication. It is highly likely that *E. coli* O157:H7 is capable of multiplying in the majority of sites in the bovine GIT to varying extents. Analysis of relative bacterial numbers over a number of animals may highlight a particular tropism but a widespread distribution would make any such conclusion difficult to reach. However, as will be described in this Chapter, sampling of long-term persistent shedders from an appropriate model system has resulted in the identification of a site of colonisation that not only accounts for the majority of faecal *E. coli* O157:H7, but also evidence of a specific tissue tropism.

The experimental challenge of weaned calves with *E. coli* O157:H7 reproduces natural carriage and does not result in clinical disease. The distribution of the challenge strain in such animals has been investigated. Brown et al. (1997) recovered *E. coli* O157:H7 from all sites sampled within the GIT, except the abomasum, with the highest recovery rate in the fore-stomachs. Cray Jr and Moon (1995) also demonstrated a ubiquitous distribution but found the highest numbers in large intestinal sites. These studies suggest a variable distribution with no specific tropism for any site within the bovine GIT. However Grauke et al. (2002) used rumen and duodenal cannulae to sample from live calves and beyond day 16 neither of these locations harboured *E. coli* O157:H7. This was despite it being present in some of the corresponding fecal samples up to day 34, implying a large intestinal location. None of these studies demonstrated any evidence of mucosal localisation or A/E lesions in persistently shedding animals.

In this study (Chapter 4), persistent shedding for over 3 weeks was consistently obtained using an oral dose of 10^9 CFU bacteria. Animals for necropsy were chosen from those shedding at 20 to 28 days post infection, with the exception of calf 307 that was necropsied at 14 days post infection. Results of the necropsies performed from these animals in addition to a naturally colonised steer identified by an IPRAVE longitudinal study and purchased from an Inverness-shire farm are described in two different forms below. The history, sampling procedures, results and results are described for each of these animals in turn. Then these results, together with those from T.E. Besser's co-habitation infection model, are summarised collectively with statistical analyses.

5.2 Individual Animal Results in Chronological Order

5.2.1 Calf 121

Calf 121 had been shedding ZAP 3 at consistently high levels (10^4 CFUg⁻¹ to 10^5 CFUg⁻¹) throughout the sampling period and was therefore a good candidate to remove from the persistence study for necropsy sampling. The day 21 post challenge faecal sample was the final faecal sample and this figure (2.3×10^5 CFUg⁻¹) is used in table 1. A faecal sample was not taken on the day of necropsy (day 23 post challenge) as it was assumed that the rectal contents would contain similar levels of *E. coli* O157:H7. The contents results were as follows, rumen (N), ileum (P), colon (50 CFUg⁻¹) and mid rectum (P). Tissue was also taken but the methodology was different from later necropsies so the results are not included in table 1 or the statistical analysis. It was concluded that the shedding level of *E. coli* O157:H7 had unfortunately dropped between day 21 and the necropsy. To prevent this uncertainty in subsequent necropsies, faecal samples were taken as close as possible prior to the euthanasia.

5.2.2 Calf 118

Calf 118 was challenged with ZAP 3. Initially the challenge was not successful with no recovery on days 14 and 17 but the organism was reacquired, possibly by re-ingestion from the environment and levels had risen to 700 and 1300 CFUg⁻¹ by days 21 and 24 respectively. On day 28 post-challenge ante-mortem faeces was taken per

rectum and the necropsy performed within an hour. The levels of *E. coli* O157:H7 in the ante-mortem faeces (2.6×10^3 CFUg⁻¹) were markedly higher than those in the GIT contents i.e. rumen (N), ileum (N), colon (N) and mid-rectum (P). This abrupt increase in *E. coli* O157:H7 levels between the mid-rectum and faeces was unlikely to be due to a general decrease in the *E. coli* O157:H7 population throughout the GIT as the necropsy was performed so soon after the faecal sample was taken. The possibility was raised that *E. coli* O157:H7 was either multiplying or colonising the GIT at a site distal to mid-rectum. This may also have been the case in calf 121, although with the two-day delay between faecal sampling and necropsy it was possible that it simply ceased shedding the organism. Tissue samples were taken but as with calf 121 they are not included in the results tables or statistical analysis.

5.2.3 Calves 323 and 310

Calves 323 and 310 are described together as their necropsies were performed on the same day with identical procedures. These animals were both shedding ZAP 198 at levels in excess of 10^4 CFUg⁻¹ throughout the period with the exception of day 21 post challenge at which time both calves shed much lower levels (430 and 90 CFUg⁻¹). Shedding levels recovered dramatically after this and by day 28 post challenge their ante-mortem faeces contained 3.3 and 3.8×10^5 CFUg⁻¹ respectively. The *E. coli* O157:H7 levels for each calf respectively were as follows, rumen (N and 45 CFUg⁻¹), ileum (both N), colon (both N) and rectum (both N). No tissue samples were collected from these animals due to logistical limitations but the levels in the gut contents were considered to be sufficient to follow up on the observations made for calves 121 and 118 approximately one year earlier (the Foot and Mouth crisis prevented animal movement in the interim). There were now at least 3 (possibly 4) out of 4 persistently colonised calves demonstrating an unexpected and unexplained pattern of colonisation. More detailed analysis of tissue samples was now necessary in order to investigate this further.

5.2.4. Calf 307

Calf 307 was challenged with ZAP196 and because of the need to follow up previous results and the risk of *E. coli* O157:H7 disappearing by the end of the sampling

period, it was necropsied at day 14. In addition to the samples taken in previous animals the distal portion of rectum together with the anal canal were removed as described in materials and methods. The distal-most 10 g of contents (in this case 10 to 20 cm from the RAJ) were taken and the *E. coli* O157:H7 in this sample was only 3×10^1 compared with 3.6×10^4 CFUg⁻¹ in the ante-mortem faeces. The remaining contents were, rumen (N), ileum (N), colon (520 CFUg⁻¹) and mid-rectum (P). In this animal there was a relatively low level of *E. coli* O157:H7 in the colon but otherwise a similar pattern to that seen in previous animals was observed. A 10 cm² tissue sample from 5 to 10cm proximal to the RAJ contained 2.5×10^3 CFUcm⁻² as determined by vortexing vigorously for 60 seconds in sterile PBS. This figure was low however when compared with that obtained for a 2cm² sample of squamous epithelium from the anal canal, 1.9×10^5 CFU cm⁻². The levels of *E. coli* O157:H7 were therefore increasing dramatically within the terminal rectum but it was still not clear as to whether this was due to tissue colonisation or a result of a rapid multiplication, perhaps in response to an environmental factor within the terminal portion of rectum such as oxygen level.

Subsequent necropsies would take more detailed tissue samples from the rectum. Contents and faeces were also used for enumeration of non-O157 *E. coli* to rule in or out the possibility of an environmental factor triggering a general multiplication of all *E. coli* strains present. In addition the ante-mortem faeces from this point, when sufficiently solid and passed naturally, was split into equal quantities (10 g) of surface and core to provide an additional means of determining the origin of the *E. coli* O157:H7. The surface levels provided an indication of bacteria contaminating stools as they passed the RAJ, in effect acting as a natural swab. The levels in the core indicated the levels of *E. coli* O157:H7 from more proximal tissue or from multiplication within the digesta.

5.2.5 Calves 299 and 295

Calves 299 and 295 were colonised with ZAP 196 and necropsied on days 24 and 25 respectively. 299 shed levels in excess of 10^5 CFUg⁻¹ throughout the colonisation period whereas 295 shed at levels of between 10^3 and 10^4 CFUg⁻¹ with the exception

of day 17 when levels dropped to be detectable only by broth enrichment. Contents contained respectively, rumen (P and N), ileum (N and N), colon (P and N), mid-rectum (150 CFUg^{-1} and N) and the distal-most contents (100 CFUg^{-1} and N). The surface layer of the ante-mortem faeces contained 2.8×10^5 and $7.0 \times 10^4 \text{ CFUg}^{-1}$ respectively in contrast to the core faeces with $3.9 \times 10^2 \text{ CFUg}^{-1}$ and N respectively. Tissue washing data from 11, 8 and 5 cm proximal to the RAJ all contained low numbers of *E. coli* O157:H7 (P to $2.1 \times 10^2 \text{ CFUg}^{-1}$) but the RAJ +2cm tissue contained increased numbers (6.2 and $6.8 \times 10^2 \text{ CFUg}^{-1}$). Much larger numbers were present on the squamous epithelium of the anal canal (8.4×10^4 and 10^5 CFUg^{-1}) suggesting that this tissue was the site of colonisation, although very few O157 positive bacteria were visible on this tissue by immunofluorescent microscopy. Subsequent necropsy results demonstrated the true site to be the tissue immediately adjacent to the RAJ. In these animals high numbers of *E. coli* O157:H7 were probably present on the rectal mucosa within 2cm of the RAJ and contaminated the surface of the squamous epithelium by being swept along in the flow of digesta.

5.2.6 The Naturally Colonised Steer - 325

325 was shedding *E. coli* O157:H7 through natural acquisition as opposed to experimental introduction of the organism. It was a 12 month old Limousin-cross steer taken from a group of 35 similar animals being raised for beef in a straw court on an Inverness-shire farm. Field epidemiology work had identified the group as being positive for *E. coli* O157:H7 based on immunomagnetic separation (IMS) of randomly sampled faecal pats. Rectal sampling of each animal within the group identified three animals shedding high levels of *E. coli* O157:H7 ($>10^4 \text{ CFU g}^{-1}$) with 13 others positive by IMS only, indicating very low levels of faecal shedding. Two of the high shedders (309 and 325) were purchased for necropsy sampling. Unfortunately 309 was negative on the day of necropsy (2 days after the initial faecal sample was taken). In 325, the ante-mortem faeces was too fluid to be split into core and surface components but the whole faecal count was $3.3 \times 10^4 \text{ CFUg}^{-1}$. Ileum, caecum, colon and mid-rectum contents were all negative for *E. coli* O157:H7 and the rumen contents were enrichment positive. The distal-most portion of rectal contents however contained $6.0 \times 10^4 \text{ CFUg}^{-1}$ but significantly this sample overlaid

the terminal 5 cm of rectal mucosa. Tissue samples were positive only on samples from the terminal 10 cm of rectum with the highest counts on the RAJ +5cm (8.3×10^4) and +2 cm (2.1×10^4) sites. The strain recovered from this animal is designated ZAP 278. It agglutinates with anti-H7 antibody (Mast-Assure), possesses genes for intimin γ , enterohaemolysin, Stx 2 and 2C and is phage type 21/28.

5.2.7 Calves 360 and 351

Calves 360 and 351 were challenged with ZAP 198 and necropsied on days 20 and 21 post-inoculation. These calves are described together since the distributions of *E. coli* O157:H7 were similar. They were challenged purely for necropsy analysis and so the shedding data obtained was limited. All GIT contents sampled contained either N or P with the exception of the rumen of calf 351 (3.3×10^1 CFUg⁻¹) and the distal-most rectal contents of both calves (4.6×10^4 and 2.1×10^3 CFUg⁻¹ respectively). The latter samples overlaid the terminal rectum adjacent to the RAJ in both animals. Faecal levels were 5.1×10^3 CFUg⁻¹ on the surface sample and P in the core sample of calf 360 and 2.4×10^3 and N in the corresponding samples of calf 351. No *E. coli* O157:H7 were recovered from the tissues of either animal other than at the terminal rectum, RAJ 3-5cm (both P), RAJ 1-3cm (2.1 and 1.1×10^3 CFUg⁻¹ respectively) and RAJ 0 to +1cm (9.8 and 1.6×10^3 CFUg⁻¹ respectively). Squamous epithelium in the region of RAJ 0 to -1cm was taken separately from these animals and the numbers of *E. coli* O157:H7 were lower than on the terminal rectal sites for both animals (5.5×10^2 and 1×10^2 CFUg⁻¹) providing additional evidence that in previous animals the high counts observed on this tissue was due to bacteria being physically moved from the terminal rectum.

5.2.8 Calf 364

Calf 364 was challenged with ZAP 198 and was notable in that it was the only shedding calf necropsied at Moredun Research Institute in which *E. coli* O157:H7 was not colonising primarily at the terminal rectum. GIT contents levels were relatively constant from the caecum to the mid-rectum. Although the levels in the surface faeces were more than 10-fold higher than the mid-rectal contents (1.4×10^3 versus 1.3×10^2 CFUg⁻¹), higher levels in the colon and the core faeces (1.1×10^3 and

8.6×10^2 CFUg⁻¹) suggests that the mid-rectal level may not have been representative of the levels in the large intestinal digesta in this individual. The small increase between surface and core faeces (approximately 1.6 fold) together with the fact that the RAJ 0 to +1cm and the RAJ 3-5cm were the only tissues in the rectum to contain *E. coli* O157:H7 (both P) suggests that a very small population may have been present on the terminal rectum, although this was probably not contributing to more than a small proportion of the faecal *E. coli* O157:H7.

5.3 Collective Necropsy Results with Statistical Analyses

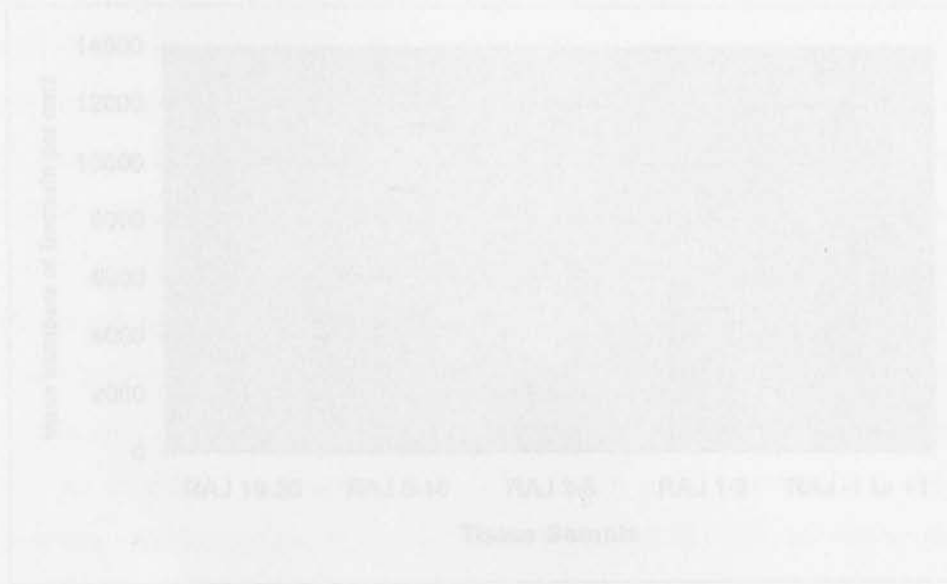
5.3.1 Distribution of *E. coli* O157:H7 in GIT Contents and Faeces at Necropsy

Necropsies were performed on experimentally and naturally colonized animals that were persistently shedding *E. coli* O157. All animals in which *E. coli* O157:H7 was detected in the ante-mortem faeces are included in this analysis. Ante-mortem faecal levels ranged from 1.4×10^3 to 3.8×10^5 CFU g⁻¹ (Table 5.1). For 12 of the 15 animals, levels of *E. coli* O157:H7 were at least 10 fold higher in the ante-mortem faeces than in any GIT contents sample. In 10 of the 15 animals the levels of *E. coli* O157:H7 in the faeces were at least a hundred fold higher than in any GIT contents sample (Table 5.1). *E. coli* O157:H7 was not recovered from the GIT contents of six rumenal, 13 ileal, 7 colonic and 7 rectal samples. Statistical analysis showed no evidence of significant differences between samples collected from the colon and the mid-rectum ($p=0.99$). However, counts were significantly higher in the faeces than in both the colonic and mid-rectal contents ($p<0.001$).

5.3.2 Distribution of *E. coli* O157:H7 on Gastrointestinal Tract Mucosa

Tissue-associated *E. coli* O157:H7 were recovered and enumerated from defined mucosal sites for 11 animals (table 5.2). Levels of mucosa-associated *E. coli* O157:H7 on the terminal 5 cm of rectum were at least 10 times higher than on any other mucosal surface examined in 9 of the 11 animals. Rumen, ileum, Peyer's patch and colon tissue, when sampled, were typically negative. Calculating the mean counts for *E. coli* O157:H7 (CFUcm⁻²) allowed differences between defined tissue sites to be compared statistically. There is clear evidence of an upward trend in counts as samples are taken closer to the RAJ, the increases being particularly strong

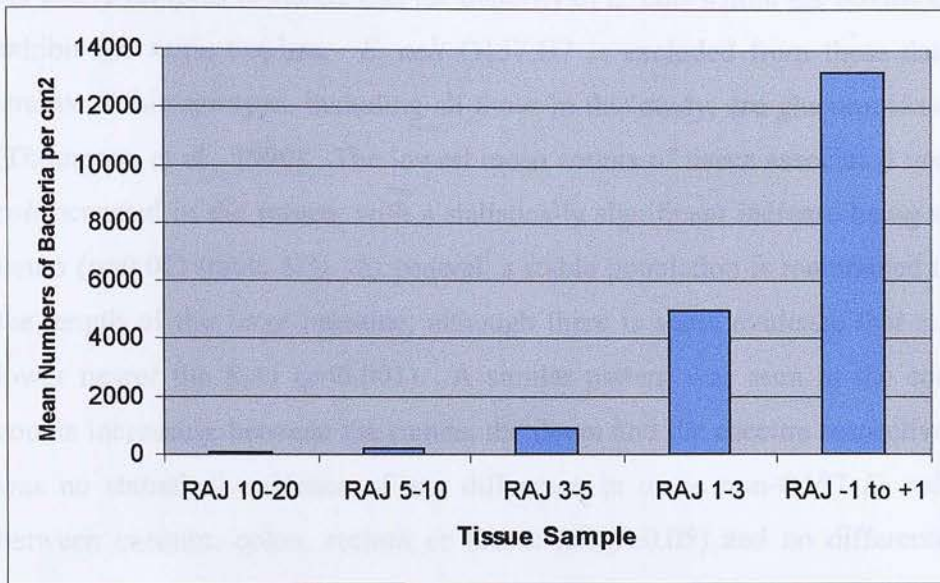
between the zones RAJ +3-5cm and RAJ +1-3cm ($p<0.001$) and between the zones RAJ +1-3cm and RAJ -1to+1cm ($p=0.001$) (fig. 5.1).



5.3.3 Distribution of *E. coli* O157:H7 Within Faeces

Freshly produced faeces from five animals were caught and directed into surface and core components. *E. coli* O157:H7 in the faeces of four animals were unevenly distributed and there was additional evidence of localisation in the terminal rectum (tables 1 and 2). For Fusa, the mean surface count was 2.1×10^6 CFUg⁻¹, approximately 1000 fold higher than the mean core count (2.3×10^3 CFUg⁻¹) (table 5.1). This difference is highly statistically significant ($p<0.001$). In contrast, there is significant differences between mean counts in the core faeces and mid-rectal contents ($p=0.02$). In calf 364, *E. coli* O157:H7 was widely distributed throughout the large intestine and was not localized specifically at the terminal rectal mucosa. There was little difference (<2 fold) between the surface and core faecal *E. coli* O157:H7 counts in this individual (table 5.1). In the remaining animals, either the necroscopy was performed before this procedure was conceived or the faecal samples were too fluid to be split into surface and core components.

Figure 5.1 Mean Levels of *E. coli* O157:H7 on Mucosa from Various Distances from the RAJ



5.3.3 Distribution of *E. coli* O157:H7 Within Faeces

Freshly produced faeces from five animals were caught and dissected into surface and core components. *E. coli* O157:H7 in the faeces of four animals were unevenly distributed and there was additional evidence of localisation to the terminal rectum (tables 1 and 2). For these, the mean surface count was 2.2×10^4 CFUg⁻¹, approximately 1000 fold higher than the mean core count (2.5×10^1 CFUg⁻¹) (table 5.1). This difference is highly statistically significant ($p < 0.001$). In contrast, there is significant difference between mean counts in the core faeces and mid-rectal contents ($p = 0.82$). In calf 364, *E. coli* O157:H7 was widely distributed throughout the large intestine and was not localised specifically at the terminal rectal mucosa. There was little difference (<2 fold) between the surface and core faecal *E. coli* O157:H7 counts in this individual (table 5.1). In the remaining animals, either the necropsy was performed before this procedure was conceived or the faecal samples were too fluid to be split into surface and core components.

5.3.4 Distribution of Non-O157:H7 *E. coli* at Necropsy

The numbers of glucuronidase positive *E. coli* were counted in a random selection of the above samples to ensure that the majority of *E. coli* within the bovine GIT do not exhibit the same tropism. *E. coli* O157:H7 is excluded from these data as most strains of this serotype, including all those in this study, are glucuronidase negative (Thompson et al., 1990). The lowest mean counts of tissue associated non-O157 *E. coli* occurred in the rumen, with a statistically significant increase being seen in the ileum ($p=0.01$) (table 5.3). In general, a stable population is maintained throughout the length of the large intestine, although there is some evidence that counts were lower nearer the RAJ ($p<0.001$). A similar pattern was seen in the contents, the counts increasing between the rumen, the ileum and the caecum respectively. There was no statistical evidence of any difference in mean non-O157 *E. coli* numbers between caecum, colon, rectum or faeces (all $p>0.05$) and no difference between surface and core faecal samples ($p=0.47$). Importantly, increases at the recto-anal junction and faecal surface were not observed. Therefore the majority of *E. coli* in the bovine gut did not exhibit the same tropism for the terminal rectum as *E. coli* O157:H7.

Calf	Strain	Milk pasteurisation challenge?	Gastrointestinal		
			Rumen	Ileum	Caecum
101	Cap 101	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
102	Cap 102	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
103	Cap 103	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
104	Cap 104	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
105	Cap 105	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
106	Cap 106	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
107	Cap 107	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
108	Cap 108	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
109	Cap 109	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
110	Cap 110	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
111	Cap 111	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
112	Cap 112	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
113	Cap 113	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
114	Cap 114	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
115	Cap 115	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
116	Cap 116	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
117	Cap 117	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
118	Cap 118	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
119	Cap 119	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}
120	Cap 120	Y	10 ^{2.5}	10 ^{3.5}	10 ^{4.5}

Table 5.1 *E. coli* O157:H7 Concentration (CFUg⁻¹) in Gastrointestinal Contents and Faeces from Shedding Cattle

Calf ^a	Strain	Day post challenge ^b	Gastrointestinal Tract Location:						Faeces:		
			Rumen	Ileum	Caecum	Colon	Mid-Rectum ^c	Whole	Surface	Core	
351	ZAP 198	21	33 ^d	0	0	0	0	0	NA ^e	2400	0
364	ZAP 198	21	0	0	670	1100	130	0	NA	1400	860
360	ZAP 198	20	P ^f	0	0	P	0	0	NA	5100	P
325	ZAP 278	NR ^g	P	0	0	0	0	0	33000	NA	NA
607	WSU2043	NR	P	550	ND ^h	750	4200	0	3200	NA	NA
609	WSU2043	NR	0	0	ND	0	0	0	5300	NA	NA
611	WSU2043	NR	P	0	ND	4700	5400	0	16000	NA	NA
612	WSU2043	NR	300	0	ND	50	150	0	30000	NA	NA
295	ZAP 196	25	0	0	ND	0	0	0	NA	70000	0
299	ZAP 196	24	P	0	ND	P	150	0	NA	280000	390
307	ZAP 196	14	0	0	ND	520	P	P	36000	NA	NA
310	ZAP 198	28	45	0	ND	0	0	0	380000	NA	NA
323	ZAP 198	28	0	0	ND	0	0	0	330000	NA	NA
118	ZAP 3	28	0	0	ND	0	P	P	2600	NA	NA
121	ZAP 3	23	0	P	ND	50	P	P	230000	NA	NA

Table 5.2 Tissue-Associated *E. coli* O157:H7 Levels (CFUcm⁻²) on Mucosa Taken from Regions of the Gastrointestinal Tract at Necropsy

Calf ^a	Strain	Day Post Challenge ^b	Mucosal Sample:											Anal SSE	
			Rumen	Ileum	Peyer's Patch	Colon	RAJ ^c 20-30	RAJ 10-20	RAJ 5-10	RAJ 3-5	RAJ 1-3	RAJ -1 to +1			
351	ZAP 198	21	0 ^d	0	0	0	0	0	0	0	0	P ^e	1100	1600	100
364	ZAP 198	21	0	0	0	P	0	0	0	0	0	P	0	P	P
360	ZAP 198	20	0	0	0	0	0	0	0	0	0	P	2100	9800	550
325	ZAP 278	NR ^g	0	0	0	0	0	0	0	0	61	83000	21000	5800	12000
607	WSU 2043	NR	0	200	ND ^h	230	ND	ND	17	250	0	33	33	33	P
609	WSU 2043	NR	0	0	ND	ND	ND	ND	0	0	0	33	50	1900	270
611	WSU 2043	NR	0	0	ND	ND	ND	ND	950	720	0	57000	300000	29000	65000
612	WSU 2043	NR	0	0	ND	ND	ND	ND	17	1600	0	3300	4000	16000	33000
295	ZAP 196	25	ND	ND	ND	ND	ND	ND	30	210	0	P	680	100000	210000
299	ZAP 196	24	ND	ND	ND	ND	ND	ND	30	45	0	15	620	84000	170000
307	ZAP 196	14	ND	ND	ND	ND	ND	ND	ND	2500	0	ND	ND	190000	370000

^a Locations of the carcass within a region 20 to 24 cm proximal to the rectum were used.

^b Values represent log₁₀ Probable Numbers (PN) rounded to 2 significant figures.

^c P, Not Applicable; other whole farms were used for enumeration at 6 week post challenge and core components.

^d P, Positive by enrichment culture but not by direct plating.

^e ND, Not Determined; *E. coli* O157:H7 was acquired at an undetermined time point.

^f ND, Not Determined.

^g RAJ, Recto-Anal Junction, numbers indicate proximal distance in cm from the unrelaxed boundary.

Table 5.3 Non-O157 *E. coli* Concentration (CFUg⁻¹) in Gastrointestinal Contents and Faeces from Cattle

Calf ^a	Gastrointestinal Tract Location:					Faeces:		
	Rumen	Ileum	Colon	Mid Rectum ^c	Whole	Surface	Core	
351	4800 ^d	>1000000	>1000000	>1000000	NA ^d	>1000000	>1000000	
364	1200	33000	54000	68000	NA	230000	230000	
360	1100	120000	280000	240000	NA	170000	180000	
325	650	11000	11000	11000	13000	NA	NA	
607	24000	500000	1000000	1000000	ND ^f	ND	ND	
609	1300	3100	55000	40000	ND	ND	ND	
611	900	400	180000	1000000	ND	ND	ND	
612	0	6200	150000	110000	ND	ND	ND	
295	820	1300	4800	8700	NA	5400	6400	
299	91	410	93000	46000	NA	45000	40000	

Key for Tables 5.1 – 5.3

- ^a Calves listed in the reverse chronological order in which necropsies were performed.
- ^b Number of days Post Challenge that the necropsy was performed.
- ^c Contents of the rectum within a region 20 to 30 cm proximal to the Recto-Anal Junction.
- ^d Values represent Most Probable Numbers rounded to 2 significant figures.
- ^e NA, Not Applicable, either whole faeces was used for enumeration or it was split into surface and core components.
- ^f P, Positive by enrichment culture but not by direct plating.
- ^g NR, Not Relevant, *E. coli* O157:H7 was acquired at an undetermined time point.
- ^h ND, Not Determined.
- ⁱ RAJ, Recto-Anal Junction, numbers indicate proximal distance in cm from this anatomical boundary

5.4 Demonstration of *E. coli* O157 Microcolonies on Terminal Rectal Epithelium by Fluorescent Microscopy.

All tissue from which *E. coli* O157:H7 was cultured directly was examined by immunofluorescent microscopy with the following exceptions: all tissue from T.E. Besser's experimental animals (607, 609, 611 and 612) and calf 307, and the RAJ 3-5 and 5-10cm samples of calf 325. Micro-colonies of *E. coli* O157 were detected using anti-O157 immunofluorescence on sections with corresponding tissue-associated bacterial counts of 2×10^3 CFUcm⁻² or greater. This included demonstration of *E. coli* O157 micro-colonies on the rectal mucosa from the naturally colonised animal (calf 325, fig. 5.2). No fluorescent bacteria were observed when either normal rabbit serum or anti-O26 antibody were used in place of the anti-O157 antibody on sections from within a series of sequential sections known to contain adherent *E. coli* O157.

Animal by animal results are described below:

5.4.1 Calves 360 & 351

Microcolonies were demonstrated on the RAJ 1-3cm and the RAJ 0 to +1cm of calf 360 but not on any tissue of calf 351 suggesting a possible lower limit for the detection of microcolonies using histological analysis of small numbers of sections of around 2×10^3 CFUcm⁻² (in these samples 50 sections were processed from each tissue).

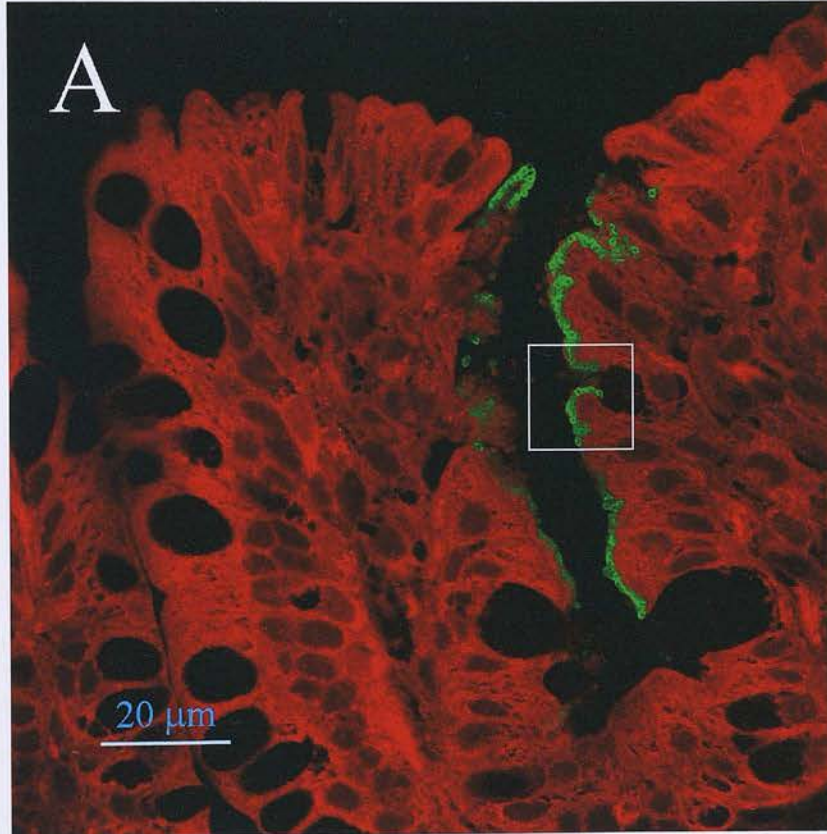
5.4.2 Calf 325

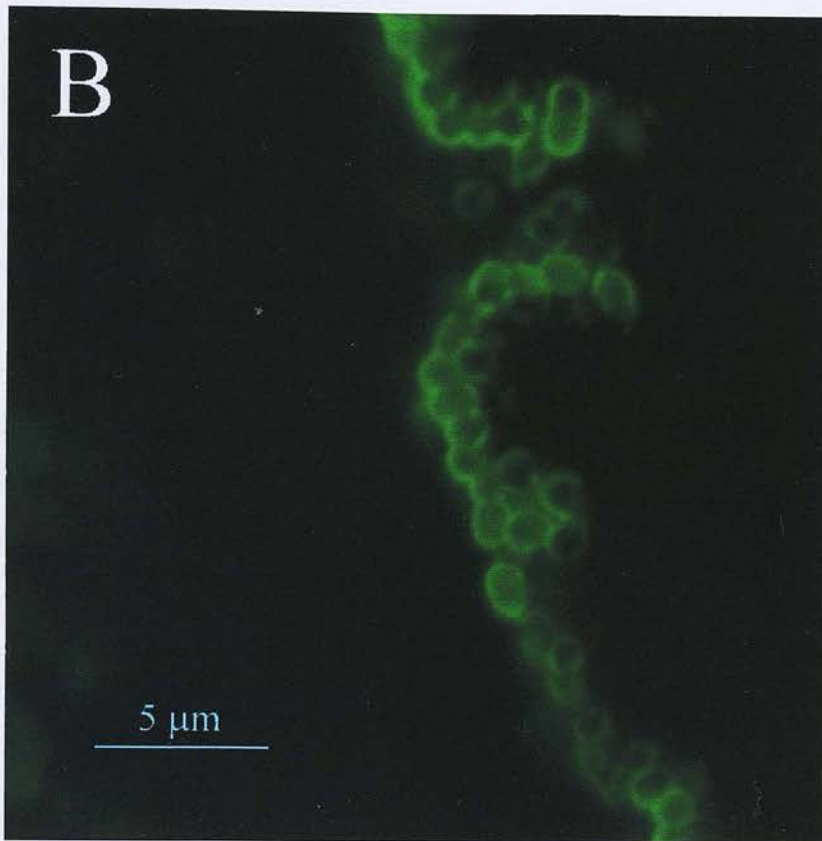
Tissue samples were positive only on samples from the terminal 10 cm of rectum and the stratified squamous epithelium of the anal canal with the highest counts on the RAJ +5cm (8.3×10^4) and +2cm (2.1×10^4) sites. The RAJ + 10 cm and +5cm sites were not assessed by immunofluorescent microscopy examination. Microcolonies of *E. coli* O157 microcolonies were present on tissue from the RAJ +2cm site and as with calf 299, a portion of columnar epithelium was taken along with the squamous epithelium containing an *E. coli* O157 microcolony (figure 5.2).

Figure 5.2 Confocal Micrographs of a Microcolony of *E. coli* O157

Within a Crypt Adjacent to the RAJ

Adherent *E. coli* O157:H7 (green) with tissue counterstained with TRITC-phalloidin (red) (63X objective) (A). A different Z-plane from a region of the same sample that has been digitally magnified by a factor of 6 using the Zeiss 510 confocal image software (B).





5.4.3 Calves 299 & 295

The rectal samples from these animals did not contain greater than 6.8×10^2 CFU g^{-1} of tissue associated *E. coli* O157:H7. The apparent lack of microcolonies on these samples was not surprising given the lower limit subsequently shown to be necessary for the observation of microcolonies (2×10^3 CFU cm^{-2}). Much larger numbers were present on the squamous epithelium of the anal canal (8.4×10^4 and 10^5) suggesting that this tissue was the site of colonisation, although very few O157 positive bacteria were visible on this tissue by immunofluorescent microscopy. As described above, subsequent necropsy results demonstrated the true site to be the tissue immediately adjacent to the RAJ. In these animals high numbers of *E. coli* O157:H7 were probably present on the rectal mucosa within 2cm of the RAJ and contaminated the surface of the squamous epithelium by being swept along in the flow of digesta. Fortunately a small portion of colorectal type mucosa was included within the RAJ 0 to -1cm sample of calf 299 for histology and microcolonies of *E. coli* O157:H7 were subsequently located on this tissue.

5.5 Demonstration of Intimate Attachment and Pedestal Formation by Electron Microscopy

The RAJ +2cm sample from calf 325 was examined one section at a time until a sample containing two individual microcolonies was identified. The remaining tissue block was then processed for TEM and ultra-thin section made at the 3 x 1mm region of the block where *E. coli* O157 microcolonies were identified. A microcolony was located on an area where epithelial cells were severely degenerated but bacteria were clearly intimately attached to membranous debris and intact pedestals were present underlying some of the attached bacteria (fig 5.3).

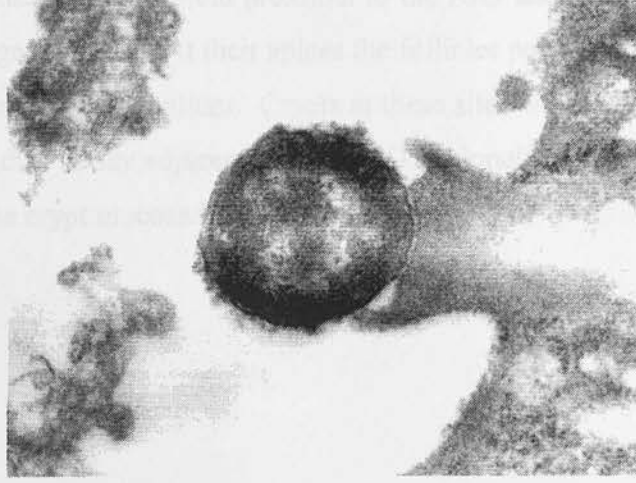
Figure 5.3 *E. coli* O157:H7 intimately attached to pedestals on Bovine Terminal R. Transmission electron micrographs of a region of terminal rectal epithelium known to be severely degenerated. Bacteria were intimately attached to cellular debris displaying morphological characteristics of pedestals.



Figure 5.3 *E. coli* O157:H7 Intimately Attached to Pedestals on Bovine Terminal Rectal Epithelium

Transmission electron micrographs of a region of terminal rectal epithelium known to contain *E. coli* O157:H7 microcolonies.

Bacteria were intimately attached to cellular debris displaying morphological characteristics of pedestals (32000x)

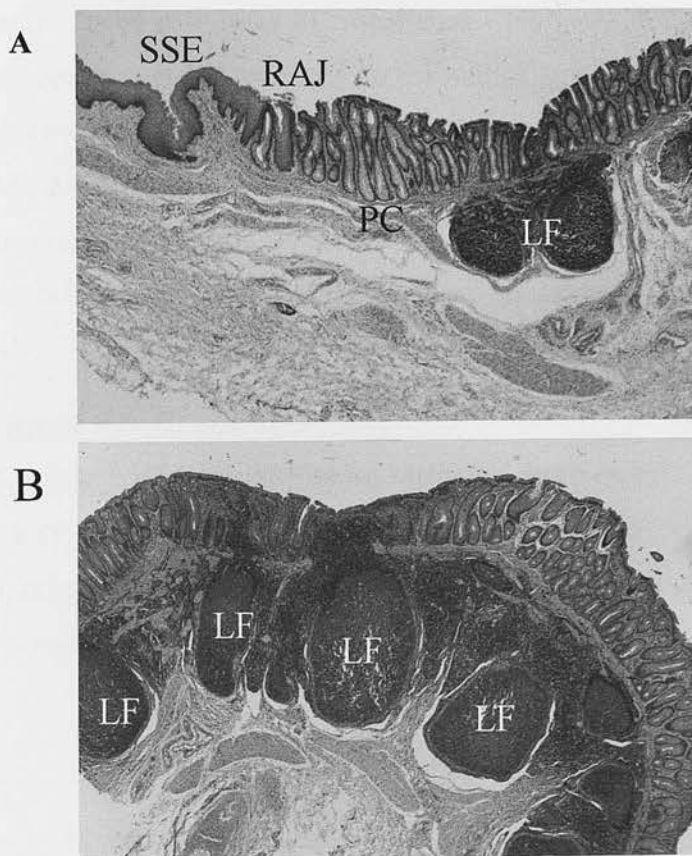


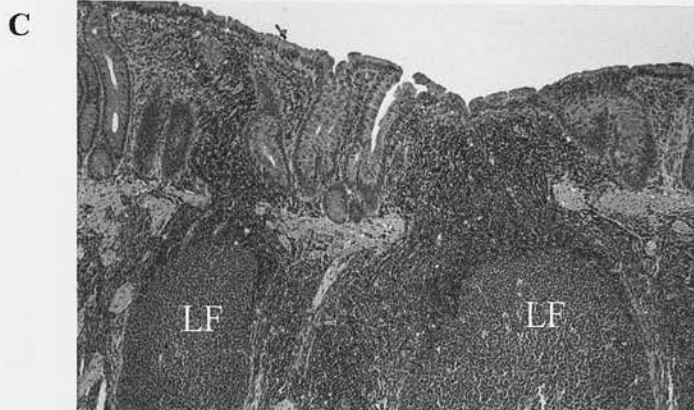
5.6 Initial Characterisation of the Mucosa of the Terminal Rectum.

In gross appearance, the smooth raised mucosa adjacent to the RAJ contrasted subtly with the folded surface of the normal absorptive rectal mucosa. These features are termed the rectal ampullae and although they coincide with the location of lymphoid follicles, they are simply the result of mucosal architecture at this site. Aggregates of lymphoid follicles formed patches evenly distributed around the circumference of the terminal rectum, an observation also made by Liebler et al. (1988). Histologically the RAJ (fig. 5.4a) is the clear junction between the stratified squamous epithelium of the anal canal with the columnar epithelial mucosa of the rectum. Large aggregates of submucosal lymphoid follicles were visible in haematoxylin and eosin stained sections of the rectal mucosa adjacent to the RAJ (Figs. 5.4 b and c). The follicle rich area extended for 2-5 cm proximal to the RAJ and appeared to increase in area in older, larger animals. At their apices the follicles penetrated the muscularis mucosa to contact areas of epithelium. Crypts at these sites were often branched and penetrated the follicles or lay adjacent to them. Occasionally the FAE lay within a recess formed by the crypt mucosa (not shown).

Figure 5.4 Haematoxylin and Eosin Stained Sections of Lymphoid Follicles in the Bovine Terminal Rectum.

The recto-anal junction (RAJ), defined as the junction between stratified squamous epithelium (SSE) and the columnar epithelium folded into pseudo-crypts (PC) that lines the large intestine (A). Two lymphoid follicles (LF) are also visible (2.5x objective) (B & C) on terminal rectal mucosa from within 1 cm of the RAJ. fig. 5.4 B demonstrates the typical density of lymphoid follicles (LF) found in this region (2.5x objective). Fig. 5.4 C highlights a lymphoid follicle in contact with an area of epithelium notable by the absence of goblet cells (10x objective).





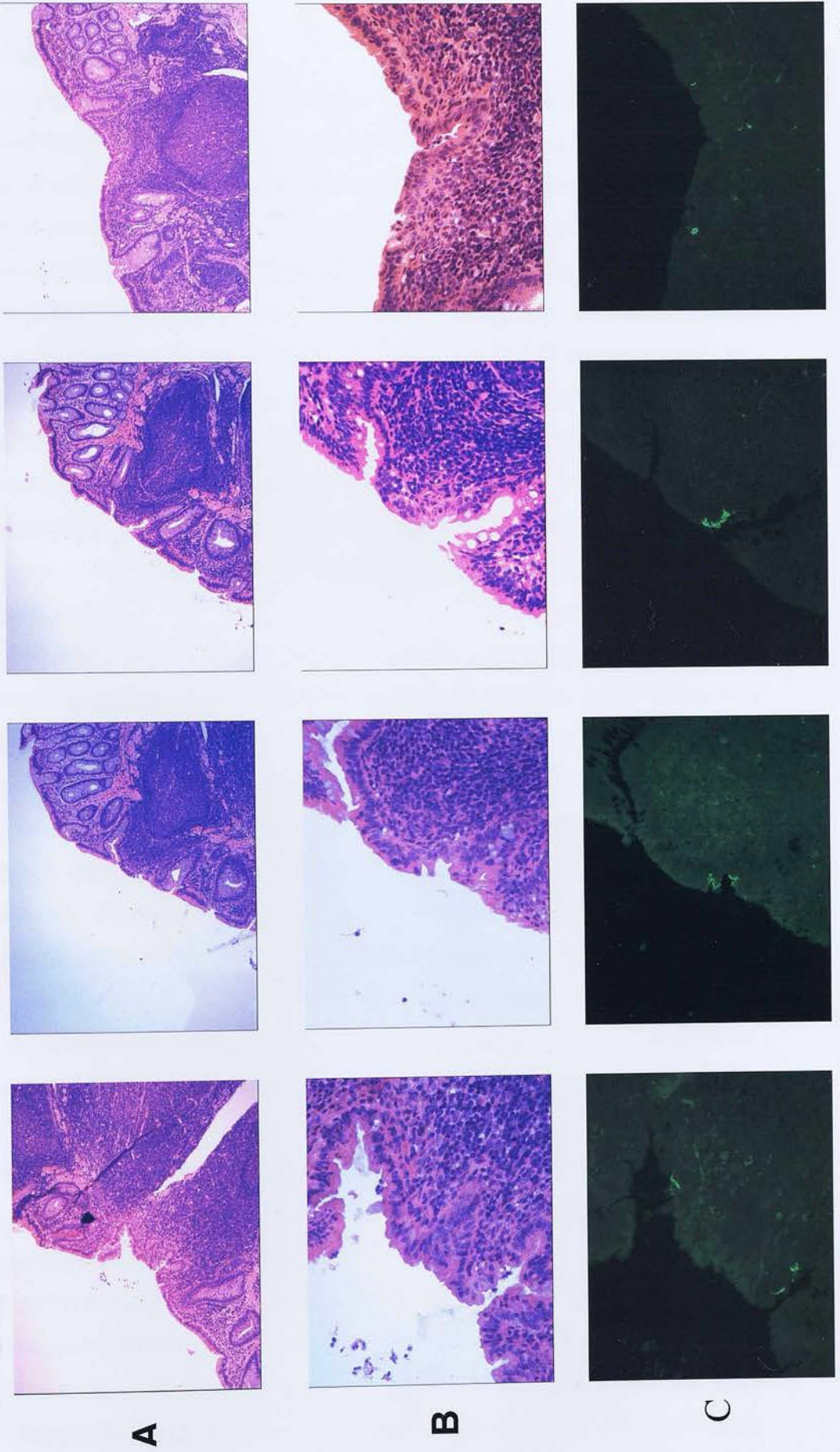
5.7 Demonstration of *E. coli* O157 Microcolonies on FAE

Figure 5.5 illustrates a number of microcolonies on FAE of the RAJ 0 to +1cm sample from calf 360. Microcolonies were more frequently observed within crypts adjacent to the follicles than on the luminal surface. Microcolonies were sometimes observed on non-FAE indicating that once localised to this region *E. coli* O157:H7 AE lesion formation can occur on non-FAE epithelium once the numbers at this location reach sufficient density. It is therefore likely that a mechanism exists that increases the efficiency of microcolony formation on the FAE at this site.

Figure 5.5 *E. coli* O157 Microcolonies on FAE at Various Sites within a Sample of Terminal Rectal Mucosa. (see over)

Each site was photographed then stained with H&E. H&E stained micrographs were taken with a 10x objective (A) and a 40x objective (B) and indirect anti-O157 immunofluorescence micrographs taken at 40x (C).

Figure 5.5



5.8 Summary

Attempts by other workers to establish the site of colonisation of *E. coli* O157:H7 in the gastrointestinal tract (GIT) of cattle have met with ambiguous and sometimes conflicting results. Considering several reports collectively, the organism can be isolated from almost every site along the length of the GIT (Brown et al., 1997; Cray Jr and Moon, 1995), particularly in animals sampled soon after receiving a high dose of bacteria (Dean-Nystrom et al., 1999). It is much harder to culture the organism from long term shedding individuals however, which presumably more closely reflect natural carriage. Early attempts to enumerate the organism in these individuals were frequently frustrated by our failure to culture bacteria from either the GIT contents or tissue, despite demonstrating considerable numbers in the faeces only a day or two prior to sampling. The possibility that *E. coli* O157:H7 does not reach significant numbers until the very last portion of the GIT was investigated by more detailed sampling of this site.

E. coli O157 was enumerated in faecal samples taken immediately before post mortem sampling and compared with the numbers in the contents from various sites along the GIT. As illustrated in table 5.1, the numbers were consistently several logs higher in the faeces than even the contents of the distal rectum. Contents from sites higher up the GIT were either very low or negative. All this suggested that *E. coli* O157 was not appearing until the final few centimetres of rectum. A key question was whether the increase in numbers was due to rapid multiplication of bacteria already present or if bacteria were being deposited on the contents during defecation. Vigorous washing of tissue samples taken at defined distances from the recto-anal junction liberated most bacteria from the tissue closest to the junction. In addition, freshly produced faeces were aseptically caught and carefully separated into surface and core components. The relative proportion of *E. coli* O157 on the surface compared with the core samples provided further evidence that bacteria were colonising the mucosal surface at the terminal rectum and that the faecal stools were being contaminated as they were passed.

Non-O157 *E. coli* were enumerated in the same samples to determine if this phenomenon was common to the *E. coli* population in general. The non-O157 *E. coli* counts did not vary significantly between large intestinal sites within the same animals. Most importantly the massive increases observed for *E. coli* O157 at the recto-anal junction and the faecal surface was not replicated by the non-O157 *E. coli*.

Immunofluorescent microscopy revealed the presence of O157 microcolonies on tissue containing more than 2×10^3 CFUcm⁻² bacteria as determined by mucosal washing. Electron microscopy confirmed that these were intimately attached. A strong association between the presence of the organism and the presence of lymphoid aggregates was observed and many microcolonies were situated on FAE, although the exact nature of the interaction is not clear.

E. coli O157 occupies an apparently unique niche in the bovine GIT. By adapting to this site it is avoiding much of the competition with other commensal microorganisms found higher up the GIT, minimising its exposure to the host immune system whilst maximising its dissemination to the environment and thus to other hosts. How this bacterium occupies this niche is an intriguing question that remains to be answered but probably involves either a site-specific adhesin or environmental control of a non-specific adhesin. The most important consequences of this finding are the implications it has for public health and the control of this infectious agent. For example, this site is accessible for accurate diagnosis and simple treatment of persistently shedding animals.

General Discussion

Chapter 6

General Discussion

6.1 Adherence to Bovine Intestinal Mucosa

The ability of *E. coli* O157:H7 to colonise the GIT of cattle is essential for its survival within the bovine population, its environment and hence to be transmitted to human hosts. Evidence that this ability depends upon adherence to and interaction with any part of the bovine intestinal epithelium was incomplete at the commencement of this body of work. Cornick et al. (2002) showed subsequently that an intimin deleted *E. coli* O157:H7 strain was impaired in its ability to persist in both sheep and cattle relative to its parent strain. The function of intimin has been well characterised and it is essential for the formation of AE lesions and intimate attachment to intestinal epithelium. A/E lesions are therefore likely to be involved in the adherence of *E. coli* O157:H7 to the bovine GIT epithelium although whether this is the primary mechanism of adherence or is a secondary event that occurs following cell contact is not certain.

One approach to investigate the role of A/E lesion formation in adherence to bovine intestinal mucosa is *in vitro* organ culture (IVOC). The experiments described compared the affinity of *E. coli* O157:H7 strains to mucosa from four intestinal sites. In one experiment, strains that varied in their ability to secrete LEE encoded proteins were compared, alongside a LEE negative deletion mutant as a negative control. *E. coli* O157:H7 strains exhibit a marked heterogeneity in their ability to secrete LEE-encoded proteins in MEM-HEPES, as first shown by McNally et al. (2001). Analysis of a relatively small number of isolates suggested that human isolates consist of a higher proportion of high secretors than bovine isolates. Since A/E lesion formation is considered to be an essential virulence factor within humans it is logical that a high proportion of isolates from human patients are those with a strong ability to produce LEE encoded proteins. The lower proportion of high Esp secretors amongst bovine isolates possibly reflects the fact that A/E is either unnecessary for persistent colonisation or a low level of A/E lesion formation is more advantageous because it is less likely to stimulate host immune responses, increasing the chance of persistence or re-colonisation. However the results of the IVOC experiment in Chapter 3 demonstrated very little correlation between intestinal mucosal affinity and LEE protein secretion status. It was not possible to identify any statistically significant difference in affinity to the intestinal sites examined, with the possible exception of the mid-rectum samples on which there was a slightly lower adherence level. Although each strain demonstrated significantly higher or lower adherence to one or

more tissue types there was no obvious pattern. When the data was analysed collectively, the high secretors had a slightly higher affinity than the low secretors to both of the large intestinal sites but the converse was true on the Peyer's patch tissue. These results imply that *LEE*-mediated adherence contributed to the affinity for large intestinal tissue. This is consistent with certain studies in which *E. coli* O157:H7 colonised the large intestine at higher levels than the small intestine in experimentally challenged calves. If A/E lesion formation mediated this colonisation, it would imply that a *LEE* encoded factor was responsible for this tropism.

Intimin has been shown to determine intestinal distribution in a number of EPEC and STEC infection models, although in *E. coli* O157:H7 the intimin type (γ) has been suggested to mediate tropism for the human Peyer's patch (Fitzhenry et al., 2002). It could have been that the differences in Esp secretion observed in MEM-HEPES were diminished once in contact with tissue, with additional external stimuli overcoming the relative suppression in the low secreting strains. However evidence that the *LEE* was not influencing mucosal affinity came from the comparison of the *LEE* deleted derivative of EDL933 (ZAP 27) which had equivalent or higher affinity for all the tissue types compared with the wild type strains, including its isogenic parent strain.

The other main IVOC experiment also provided evidence against the contribution of the *LEE* to intestinal mucosal affinity. Intimin deleted derivatives of two different AEEC serotypes were not impaired in their mucosal affinity relative to their isogenic parent strains and complementation with two different cloned intimin hybrids did not enhance affinity. These results do not conflict with those of Dean-Nystrom et al. (1999) or Cornick et al. (2002), who demonstrated the ability of *E. coli* O157:H7 to form A/E lesions and mediate colonisation respectively *in vivo*, or those of Baehler and Moxley (2000) or Phillips et al. (2000) who demonstrated A/E lesions on bovine intestinal IVOC. Instead they are simply the consequence of an alternative method of assessing bacterial-epithelial affinity. Many of the samples contained occasional putative A/E lesions (although none were confirmed as such by TEM) but these were not attributed with any greater significance than individual adherent bacteria. The absence of any impact on the overall affinity of any *LEE* related factor suggests that these were not contributing significantly to initial adherence but instead other factors, possibly an adhesin or possibly the inherent physical surface properties

of these organisms resulted in them associating with the epithelial surface in the numbers observed.

Considering the number of bacteria added to each explant, the level of epithelial-associated bacteria was surprisingly low, regardless of the mechanism of adherence. This is consistent with various observations in the literature, such as: 1. A/E lesions have never previously been reported in naturally colonised animals; 2. A/E lesions are very sparse even when large numbers of organisms are exposed to *in vivo* tissue in experimentally challenged cattle (Dean-Nystrom et al., 1999) and ligated gut loops (Stevens et al., 2002); 3. no effect on mucosal affinity to bovine tissue explants has subsequently been reported for an intimin deleted strain in another study (Cookson and Woodward, 2003).

The limited number of strains used in this IVOC experiment, i.e. 2 low versus 3 high *LEE* secretors, may have resulted in detecting differences between strains caused by factors other than *LEE* protein secretion status. Performing the experiment on a larger number of strains or the use of isogenic low and high secretors may have alleviated this problem. Neither alternative was possible due to the limited number of IVOCs that can be set up simultaneously in the case of the former, and our lack of understanding of the molecular basis for *LEE* protein secretion heterogeneity in the case of the latter. It is possible that the culture conditions were not favourable for AE lesion formation, perhaps due to the oxygen-enriched atmosphere. Baehler and Moxley (2000) showed that AE lesion formation on bovine IVOC was improved without oxygen enrichment. However the results discussed in Chapter 5 may provide an explanation as to why *E. coli* O157:H7 may not form AE lesions efficiently on tissue from these sites, i.e. the tropism of *E. coli* O157:H7 for the terminal rectum relative to the rest of the GIT.

The *LEE* negative strain ZAP 27 was included in this experiment as a negative control for *LEE* mediated adherence. As discussed above, the adherence of ZAP 27 to all tissue types examined was not significantly lower than the other strains with the possible exception of terminal ileum. Either *LEE* mediated adherence was not contributing to the observed adherence levels or the absence of the *LEE* in ZAP 27 was being compensated for by another factor. On the Peyer's patch ZAP 27 adhered at higher levels than its wild type parent, ZAP 26. The magnitude of this difference was far higher than between any other two strains on the same tissue type within this experiment, approximately 4-fold. Unlike the comparison between other strains,

because ZAP 26 and 27 are isogenic, it is likely that the observed difference was a genuine effect of the absence of the *LEE*, however complementation would be required to confirm this. It is possible that the *LEE* represses the expression of alternative adherence factors. Although further work would be necessary to confirm this, work by Elliott et al. (2000) provides the basis for a plausible hypothesis. Their work demonstrated that the deletion of the *LEE* encoded regulator (*LER*) in *E. coli* O157:H7 results in the expression of fimbriae structurally similar to the long polar fimbriae (LPF) of *Salmonella typhimurium*. *LER* is deleted along with the *LEE* and any repressive effects are therefore also absent in ZAP 27. The possibility that ZAP 27 was expressing fimbriae was investigated using TEM of bacteria negatively stained with phosphotungstic acid. Within a sample of ZAP 27, two bacteria possessed long, straight fimbriae located at the poles, structurally resembling LPF of *S. typhimurium*. These fimbriae were not seen in the other strains examined, although numerous short structures, presumably EspA filaments, were seen. The hypothesis that LPF derepression was the reason for the enhanced adherence of ZAP 27 on Peyer's patch epithelium is strengthened by the fact that in *S. typhimurium*, LPF mediate a tropism for the murine Peyer's patch. However subsequent work by Torres *et al* (Torres et al., 2002) has shown that one of the two LPF like fimbrial gene clusters in EDL933 mediates a localised adherence pattern, presumably by inter-bacterial adhesion analogous to bundle-forming pili of EPEC.

An important outcome of the assessment of *LEE* secreted protein status by indirect immunofluorescence was the discovery that the levels of secreted protein correlated with the proportion of bacteria within the population that expressed EspA filaments which in turn correlates with the proportion expressing intimin. The observation that these factors are both phase variable and co-regulated are a major step forward in the understanding of *LEE* gene regulation and what dictates whether a strain is a high or low secretor. This is the subject of ongoing research within the ZAP lab and ultimately may prove useful in identifying a sub-population of *E. coli* O157:H7 strains that represent the greatest risk to humans.

6.2 Colonisation and Persistence of *E. coli* O157:H7 in Calves

In Chapter 4 groups of calves were challenged with *E. coli* O157:H7 and once established the organism was able to persist for over 3 weeks. One observation was that in some instances the challenge strain was not detected in the first day following

inoculation and in the case of the Walla 1 (ZAP 196) challenged group in CCE3, re-challenge with the same organism resulted in all the calves becoming colonised. The possibility of a proportion of animals not being colonised must be taken into consideration when designing future colonisation experiments.

With the exception of ZAP 193 all the *E. coli* O157:H7 strains examined were capable of colonising and persisting for up to 28 days in conventionally reared, weaned calves using the system described. Importantly a Stx negative strain colonised at least as effectively as its Stx positive co-strain validating its use in future studies in containment level 2, which enables much larger numbers of calves to be incorporated into future experiments, which is essential given the large inherent variation in shedding levels of this organism, even between daily samples from the same animal. This result suggests that there is no absolute requirement for Stx in the persistent colonisation of calves by *E. coli* O157:H7, however it may still play a subtle role in mediating an evolutionary advantage by for example, down-regulating immune responses to either enhance the duration or level of shedding or to facilitate re-colonisation. Any such subtle effect would not be detected in these model systems due to the small numbers of animals and the large variation in colonisation pattern within each group.

There are various explanations for the poor colonisation ability of O26. The O26 serogroup may be diverse and consist of strains adapted for different host species and ages. For example, the challenge strain may have adapted to cause a relatively short-term, intense colonisation of compromised or immunologically naïve young animals which would result in clinically apparent disease. The calves challenged may have experienced sub-clinical pathology that was transiently evident in one individual. Challenge of younger animals e.g. 1 week old, may have resulted in detectable disease. The use of an O26 isolate from an older animal may have resulted in more persistent colonisation of the weaned calves since such a strain may have been better adapted to the age of calves used in these experiments. If however the colonisation pattern observed was in fact a typical representation of *E. coli* O26 colonisation, then it has implications for *E. coli* O157:H7, the duration of colonisation of which appears to be far superior. A relevant observation from one group, that is currently unpublished, is that despite the fact that O26 serotypes are common within Scottish cattle, very few actually possess both Stx and intimin, which would explain why it is

far less frequently associated with human disease than *E. coli* O157:H7 and possibly why it may possess additional virulence factors and colonise in a different manner.

6.3 Location of *E. coli* O157:H7 Within the Bovine GIT

Any tropism of a micro-organism for a specific region or component of the GIT may be masked by the ubiquitous distribution of the organism that occurs as a result of faecal passage and mixing. When localising bacteria within the GIT it is essential to examine the relative numbers within each GIT compartment as well as region. For example, if there are adherent bacteria on the epithelial cell surface and the corresponding digesta contains high numbers of the same organism, it is unclear which population is responsible for the presence and persistence of the organism and in practice multiplication could occur in both. This is further confounded by the fact that the numbers generated are not directly comparable as they are derived by different methods. In such instances, one approach to this problem would be to compare the ratio of tissue-associated bacteria to digesta-associated bacteria at different sites. Comparison of these ratios with those of related commensal strains could also be used to distinguish between colonised and contaminating bacteria. In such instances the ability of strains deleted in, for example, a known adherence factor, to colonise a host relative to the wild type or complemented strain can be performed to demonstrate the importance of adherence. In the case of *E. coli* O157:H7 the deletion of intimin has been shown to compromise its ability to colonise both the bovine and ovine (Cornick et al., 2002) host, and therefore adherence to the intestinal epithelium is almost certainly an essential component of colonisation and persistence.

The results in Chapter 5 demonstrate that *Escherichia coli* O157:H7 exhibits a novel tropism for the terminal rectum in the bovine host. In almost all persistently colonised animals, the majority of tissue-associated bacteria were identified in a region within five, or in many cases three centimetres proximal to the recto-anal junction (RAJ, fig. 3A). The tropism for the terminal rectum was demonstrated in orally inoculated calves, calves colonised by co-habitation with an animal shedding *E. coli* O157:H7 and in a naturally colonised shedding animal identified on a commercial beef farm. This tropism was demonstrated for four *E. coli* O157:H7 strains, ZAP 3, ZAP 196/198, ZAP278 and WSU2043, indicating that this is a characteristic of the O157:H7 serotype and not of any particular strain.

The mucosa at this site contrasts from that of the majority of the large intestine in that there is a high density of lymphoid follicles (Liebler et al., 1988). This was observed in the tissue samples from the animals described in Chapter 5 and has subsequently been confirmed in abattoir-derived tissue. The change in epithelial type coinciding with the localisation of a bacterial strain indicates a possible tropism relying upon an interaction of a bacterial factor with a host factor distributed predominantly on FAE. Although microcolonies were seen on colorectal type epithelium of the terminal rectum, many microcolonies were located on FAE. It is therefore not entirely clear whether *E. coli* O157:H7 targets the FAE as an initial adherence step with colonisation of the region leading to non-FAE being incidentally colonised, or if an alternative mechanism is responsible. Such mechanisms could include an environmental stimulus that up-regulates expression of an initial adherence factor, or a tropism for the mucus at this site.

The presence of lymphoid tissue at this site has been described for both neonatal calves (Liebler et al., 1988) and sheep (Sedgmen et al., 2002). The former describes the distribution of lymphoid follicles throughout the bovine large intestine and focuses on an area at the junction between the proximal and spiral colon that is particularly rich in lymphoid tissue. It is possible that in certain animals within this study (e.g. calves 611 and 307) this area was colonised by *E. coli* O157:H7 although because this was not known about during sampling it simply appeared that there was colonisation of the entire colon with dissemination to all down-stream sites. This site was certainly less frequently colonised by *E. coli* O157:H7, if at all, than the terminal rectum and the reason for this may be differences in receptor expression. Alternatively the numbers reaching this site may typically be so low that colonisation hardly ever occurs. The organism will have additional opportunity to multiply in the lumen between the proximal colon and the terminal rectum, which would increase the probability of bacteria reaching the FAE at this site.

An implication of this is that reasonable numbers of organism must first reach FAE for it to stand a chance of becoming colonised. This would be consistent with the fact that a large dose of bacteria is required to guarantee the successful colonisation of calves and smaller doses result in a lower proportion of calves being colonised. Once shedding from a proportion of animals within a group, *E. coli* O157:H7 may re-populate the GIT by re-ingestion from a contaminated environment, an event that may have occurred in certain animals in this study (e.g. calf 118, Table

4.2). Thus, mechanisms that contribute to the general colonisation of the GIT may be essential for initial colonisation and in re-colonising the terminal rectum when the population at this site is lost. This would also explain why lower challenge doses are unreliable at creating persistent colonisation. If the colonisation at the terminal rectum was unstable it may account for the fluctuations in numbers of shed *E. coli* O157:H7 observed in our shedding data and reported in other studies (Cray Jr and Moon, 1995). It almost certainly contributes the major portion of shed organisms in persistently colonised animals but a dependence upon the organism getting to the site in reasonable numbers would mean that it would be acting an amplification step.

The factors that drive this tropism are unknown since bacterial tropism for this site has not been previously described. It is likely to be based on the interaction between a bacterial adherence factor and an epithelial cell receptor, access or expression of which occurs predominantly in the terminal rectum. It is also likely that any such target is associated with the abundant presence of FAE at this site. Further characterisation is necessary to determine how similar this site is to lymphoid follicle rich tissue at other sites and in other species. In humans, lymphoid follicles in the large intestine are solitary and diffusely distributed. If the factor that mediates tropism to the bovine terminal rectum also mediates adherence to human large intestinal FAE then this would lead to extensive colonisation of the gut surface leading to disease.

An alternative explanation for the observed distribution is that *E. coli* O157:H7 does not possess an FAE specific adherence factor and has instead evolved to be poor at adhering to intestinal epithelium, making use of the inherent ability of M-cells to bind to and phagocytose microscopic particles. Once at the M-cell surface phagocytosis is prevented by the cytoskeletal re-arrangement that occurs as a result of A/E lesion formation. The population builds up on the terminal rectum and bacteria may disseminate locally so that microcolonies can be observed even on non-FAE epithelium. The absence of *E. coli* O157:H7 on the Peyer's patch of the ileum conflicts with this hypothesis and like the hypothesis of a ubiquitous FAE adherence factor, requires the caveat that the *E. coli* O157:H7 population must rarely be high enough to result in effective colonisation of the FAE at the more proximal sites.

Other enterobacteriaceae e.g. *Salmonella*, *Listeria*, *Shigella* and *Yersinia* (Jensen et al., 1998; Vazquez-Torres and Fang, 2000) have well described tropisms

for Peyer's patch FAE in various hosts. There is some evidence that intimin- γ which is expressed by EHEC O157:H7 mediates a specificity for FAE of human Peyer's patches (Fitzhenry et al., 2002). Cornick *et al* have demonstrated the importance of intimin to the persistence of *E. coli* O157:H7 in ruminant colonisation and the presence of A/E lesion forming microcolonies at the terminal rectum in this study supports a role for intimate attachment at the terminal rectum of the bovine host. Intimin may therefore mediate the tropism in addition to being required for the intimate attachment that subsequently occurs.

The tropism for a small region of the GIT may have evolved to minimise contact with host tissue whilst maintaining persistent shedding in the faeces at relatively high levels. This would limit the induction of both innate immune responses, leading to clearance of the organism and adaptive immune responses that would limit subsequent colonisation. By targeting tissue modified for antigen sampling there may also be down-regulation of such immune responses by exported bacterial factors. Non-O157:H7 EHEC or EPEC strains lacking this tropism may colonise other regions of the GIT more effectively but in doing so stimulate immune responses that lead to earlier clearance of the organism and possibly disease.

E. coli O157:H7 was present at the terminal rectum in the naturally colonised animal (325, Tables 5.1 & 5.2) on which mucosally adherent *E. coli* O157:H7 were demonstrated. This animal was taken from a straw court containing 35 cattle, 16 of which were fecal positive for *E. coli* O157:H7 by immunomagnetic separation, but of these only three individuals were shedding high numbers ($>10^4$ CFUg⁻¹). One hypothesis is that among an *E. coli* O157:H7 positive group of animals there are a small number of "super-shedders" that greatly enhance transmission and persistence within a herd. The data in Chapter 5 data shows that high shedding results principally from colonisation at the terminal rectum and therefore rectal colonisation is likely to be a pre-requisite for super-shedders on the farm. An ongoing abattoir study is providing evidence in support of this hypothesis.

The apparent sudden emergence of *E. coli* O157:H7 as a major human food-borne pathogen may be related to the acquisition of the factor or factors that mediate the tropism to the terminal rectum. This may simply be a result of increasing the prevalence within the bovine population and hence increasing the risk of human

exposure or may also be due to these factors enhancing the organism's ability to colonise and/or cause disease within humans.

6.4 Summary: A Concept of *E. coli* O157:H7 Biology Within the Bovine Host Based on Current Literature and the Results of this Thesis

In order to survive within the bovine population and its environment, *E. coli* O157:H7 must be able to multiply at some stage in its life cycle. This occurs within the bovine GIT and colonisation results in shedding of the organism within faeces for up to several weeks at levels of up to 10^5 CFUg⁻¹. Once excreted it is ingested by other animals within the group that may also become persistently colonised. Numbers ingested may be low but sufficient numbers of animals exposed to the organism over a period of time will inevitably result in some of them becoming colonised. Thus the organism remains within the group for a prolonged period, even though the individuals that are shedding the organism may change over time. Eventually the organism is lost from the high shedders before any new individuals become colonised and it ceases to colonise the group. Movement of colonised individuals can result in the sudden loss or appearance of the organism from a group and is a key factor in the dissemination of the organism throughout the bovine population. The ability to identify and treat such individuals prior to transportation would therefore contribute enormously to the control of the organism.

In order to colonise the bovine GIT at the level and for the duration required to be maintained within a herd, adherence to the intestinal mucosa via the well-described mechanism of A/E lesion formation is required. Instead of a widespread diffuse distribution of A/E lesions the organism appears to establish long-term persistence by colonising a very small region of the mucosal surface at a strategic location, the terminal rectum. This allows efficient surface contamination of faecal stools as they are released whilst minimising the number of organisms exposed to the host immune system. It would also concentrate secreted factors such as Stx, EspP, Lif, EspF or MAP that may down-regulate innate host immune responses, that would otherwise limit the duration or level of shedding, or adaptive immune responses, that may prevent subsequent re-colonisation.

Ironically *E. coli* O157:H7 appears to be targeting tissue that has evolved to sample antigenic material and present it to the immune system. This would seem disadvantageous but several other bacteria have adapted mechanisms to target FAE,

mostly to facilitate invasion, and if the organism can manipulate host immune responses, then this is a logical tissue to target. The process of cytoskeletal rearrangement and pedestal formation that occur at the sites of attachment may prevent uptake of the attached organisms by M-cells. It is likely that a non-*LEE* encoded factor is acting as the initial adherence factor. Tightly controlled regulation of such a factor may also be crucial both to prevent colonisation of other FAE rich sites and to minimise immune responses to what must be a surface exposed antigen. Such control could be achieved by the mechanism of phase variation that has been described for other adhesins, e.g. type 1 fimbriae (Eisenstein, 1981). Phase variable adhesins promoters are known to “cross talk” (Xia et al., 2000) and such an adhesin in *E. coli* O157:H7 may have a similar relationship with *LEE4* proteins that are also phase variable within this serotype. Vaccination with a normally phase-off adhesin factor is likely to result in the prevention of this tissue tropism and thus reduce the levels of *E. coli* O157:H7 within its reservoir host.

6.5 Future Work

This study has raised several avenues for further study including:

1. Confirm the importance of terminal rectal localisation to the existence of “super shedders” of *E. coli* O157:H7
2. Determine if FAE is in fact the basis for terminal rectal localisation and if so what cell type is being targeted
3. Determine the significance of other gut associated lymphoid tissue to the colonisation of *E. coli* O157:H7 in the bovine GIT
4. Determine the molecular basis for terminal rectal localisation
5. Develop basic strategies to detect and control the organism at this site

Adair-Bell, J., Frankel, G., Bels, E., Gonzalez, A. G., Trabulsi, L. R., Douce, G., Kautton, S., and Dougan, G. (1998) Detection of immune sites, beta, gamma, and delta, for immune derivatives expressed by attaching and effacing microbial pathogens. *Journal of Clinical Microbiology* 36(3): 662-668.

Aladif, S., Itoh, K., Tazji, A., Ito, R., Rishi, H., Hayakawa, T., Ihara, J., Aho, T., Haseki, M., Mori, T., and Nakamura, Y. (1994) A Severe Outbreak of Hemorrhagic Colitis and Hemolytic Syndrome Associated With Enterohemorrhagic O157:H7 in Japan. *European Journal of Pediatrics* 151(9): 650-653.

Alizon, L., Sillrat, A., and Thompson-Carter, R. M. (1998) Genetic heterogeneity of *Escherichia coli* O157:H7 in Scotland and its utility in strain subtyping. *European Journal of Clinical Microbiology and Infectious Disease* 17: 544-548.

Chapter 7

Ashwood, E., Lewis, J., and Barry, T. G. (1992) The adherence of verotoxin-producing *Escherichia coli* to rabbit intestinal cells. *Journal of Medical Microbiology* 37(2): 309-313.

Budler, A. A., and Morley, R. A. (2002) Effect of culture conditions on *Escherichia coli* O157:H7-mediated attaching-effacing lesion in a bovine large intestinal mucosal explant model. *Journal of Microbiology Letters* 212: 109-116.

Budler, A. A., and Morley, R. A. (2003) Enterohemorrhagic *Escherichia coli* O157:H7 induced attaching-effacing lesions in large intestinal mucosal explants from adult cattle. *Journal of Microbiology Letters* 180: 229-242.

References

Baker, D. R., Morley, R. A., and Dougan, G. (2000) Variation in virulence in the enterohemorrhagic *Escherichia coli* O157:H7 epidemic: an analysis of bovine and human origins. *Archives of Medical Biology* 412: 33-48.

Balows, A., Hausler, W. J., Herrington, E. V., Tenover, H. C., and Tully, H. J. (1991). *Manual of clinical microbiology*, 5th Edition (Washington, D.C.: ASM Press).

Barrick, E. N., Carle, L., and Griener, R. (1998) Comparison of 14 PCR systems for the detection and subtyping of the genes in Shiga-toxin-producing *Escherichia coli*. *Research in Microbiology* 149(7): 437-442.

Bathelot, M., Prayman, B., Daniel, S., Rapp, S., Chomartin, L., Blomberg, G., Dougan, G., Frankel, G., and Matthews, S. (2005) Structural basis for recognition of the translocated intimin receptor (Tir) by immune fluid enteropathogenic *Escherichia coli*. *EMBO Journal* 19(11): 2452-2464.

Bayer, M. E., and Walsh, R. A. (1996) Characterization of an RT4 toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* 64(1): 167-175.

AduBobie, J., Frankel, G., Bain, C., Goncalves, A. G., Trabulsi, L. R., Douce, G., Knutton, S., and Dougan, G. (1998). Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. *Journal of Clinical Microbiology* **36**(3): 662-668.

Akashi, S., Joh, K., Tsuji, A., Ito, H., Hoshi, H., Hayakawa, T., Ihara, J., Abe, T., Hatori, M., Mori, T., and Nakamura, T. (1994). A Severe Outbreak of Hemorrhagic Colitis and Hemolytic Syndrome-Associated With *Escherichia-Coli* O157:H7 in Japan. *European Journal of Pediatrics* **153**(9): 650-655.

Allison, L., Stirrat, A., and Thompson-Carter, F. M. (1998). Genetic heterogeneity of *Escherichia coli* O157:H7 in Scotland and its utility in strain subtyping. *European Journal of Clinical Microbiology and Infectious Disease* **17**: 844-848.

Ashkenazi, S., Larocco, M., Murray, B. E., and Cleary, T. G. (1992). The adherence of verocytotoxin-producing *Escherichia coli* to rabbit intestinal cells. *Journal of Medical Microbiology* **37**(5): 304-309.

Baehler, A. A., and Moxley, R. A. (2002). Effect of culture conditions on *Escherichia coli* O157:H7-mediated attaching-effacing lesions in a bovine large intestinal mucosal explant model. *FEMS Microbiology Letters* **212**: 107-110.

Baehler, A. A., and Moxley, R. A. (2000). *Escherichia coli* O157:H7 induces attaching-effacing lesions in large intestinal mucosal explants from adult cattle. *FEMS Microbiology Letters* **185**: 239-242.

Baker, D. R., Moxley, R. A., and Francis, D. H. (1997). Variation in virulence in the gnotobiotic pig model of O157:H7 *Escherichia coli* strains of bovine and human origin. *Adv Exp Med Biol* **412**: 53-58.

Balows, A., Hausler, W. J., Herrmann, K. L., Isenberg, H. D., and Shadomy, H. J. (1991). *Manual of clinical microbiology*, 5th Edition (Washington, D.C.: ASM Press).

Bastian, S. N., Carle, I., and Grimont, F. (1998). Comparison of 14 PCR systems for the detection and subtyping of stx genes in Shiga-toxin-producing *Escherichia coli*. *Research in Microbiology* **149**(7): 457-472.

Batchelor, M., Prasanna, S., Daniell, S., Reece, S., Connerton, I., Bloomberg, G., Dougan, G., Frankel, G., and Matthews, S. (2000). Structural basis for recognition of the translocated intimin receptor (Tir) by intimin from enteropathogenic *Escherichia coli*. *EMBO Journal* **19**(11): 2452-2464.

Bauer, M. E., and Welch, R. A. (1996). Characterization of an RTx toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **64**(1): 167-175.

- Baumler, A. J., and Heffron, F.** (1995). Identification and sequence analysis of *lpfABCDE*, a putative fimbrial operon of *Salmonella typhimurium*. *Journal of Bacteriology* **177**(8): 2087-2097.
- Bennet-Guerrero, E., McIntosh, T.J., Barclay, G.R., Snyder, D.S., Gibbs, R.J., Mythen, M.G. and Poxton, I.R.** (2000). Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine. *Infection and Immunity* **68**(11): 6202-6208.
- Besser, R. E., Lett, S. M., Weber, J. T., Doyle, M. P., Barrett, T. J., Wells, J. G., and Griffin, P. M.** (1993). An Outbreak of Diarrhea and Hemolytic Uremic Syndrome From *Escherichia-Coli* O157-H7 in Fresh-Pressed Apple Cider. *Journal of the American Medical Association* **269**(17): 2217-2220.
- Besser, T. E., Richards, B. L., Rice, D. H., and Hancock, D. D.** (2001). *Escherichia coli* O157:H7 infection of calves: infectious dose and direct contact transmission. *Epidemiology and Infection* **127**: 555-560.
- Bettelheim, K. A.** (2000). Role of non-O157 VTEC. *Journal of Applied Microbiology* **88**(suppl.): 38S-50S.
- Beutin, L., Montenegro, M.A., Orskov, I., Orskov, F., Prada, J., Zimmermann, S. and Stephan, R.** (1989). Close association of verotoxin (Shiga-like toxin) production in strains of *Escherichia coli*. *Journal of Clinical Microbiology* **27**(11):2559-2564.
- Bielaszewska, M., Janda, J., Blahova, K., Minarikova, H., Jikova, E., Karmali, M. A., Laubova, J., Sikulova, J., Preston, M. A., Khakhria, R., Karch, H., Klazarova, H., and Nyc, O.** (1997). Human *Escherichia coli* O157 : H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology and Infection* **119**(3): 299-305.
- Boerlin, P., McEwen, S.A., Boerlin-Petzold, F., Wilson, J.B., Johnson R.P. and Gyles, C.L.** (1999). Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *Journal of Clinical Microbiology* **37**(3): 497-503.
- Booher, S. L., Cornick, N. A., and Moon, H. W.** (2002). Persistence of *Escherichia coli* O157 : H7 in experimentally infected swine. *Veterinary Microbiology* **89**(1): 69-81.
- Borczyk, A. A., Karmali, M. A., Lior, H., and Duncan, L. M. C.** (1987). Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *The Lancet* **1**(8524): 98.
- Boyd, B., and Lingwood, C.** (1989). Verotoxin receptor glycolipid in human renal tissue. *Nephron* **51**(2): 207-210.

- Brown, C. A., Harmon, B. G., Zhao, T., and Doyle, M. P.** (1997). Experimental *Escherichia coli* O157:H7 carriage in calves. *Applied and Environmental Microbiology* **63**: 27-32.
- Brown, H., and Prescott, R.** (1999). *Applied Mixed Models in Medicine* (Chichester: Wiley).
- Brunder, W., Schmidt, H., and Karch, H.** (1997). EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Molecular Microbiology* **24**(4): 767-778.
- Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J., and Blattner, F. R.** (1998). The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Research* **26**(18): 4196-4204.
- Camilli, A., Beattie, D. T., and Mekalanos, J. J.** (1994). Use of Genetic-Recombination As a Reporter of Gene-Expression. *Proceedings of the National Academy of Sciences of the United States of America* **91**(7): 2634-2638.
- Cerqueira, A. M. F., Guth, B. E. C., Joaquim, R. M., and Andrade, J. R. C.** (1999). High occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State, Brazil. *Veterinary Microbiology* **70**(1-2): 111-121.
- Chapman, P. A.** (2000). Sources of *Escherichia coli* O157 and experiences over the past 15 years in Sheffield, UK. *Journal of Applied Microbiology* **88**: 51S-60S.
- Chapman, P. A., and Siddons, C. A.** (1996). Evaluation of a commercial enzyme immunoassay (EHEC-Tek) for detecting *Escherichia coli* O157 in beef and beef products. *Food Microbiology* **13**(2): 175-182.
- Chapman, P. A., Siddons, C. A., Malo, A. T. C., and Harkin, M. A.** (2000). A one year study of *Escherichia coli* O157 in raw beef and lamb products. *Epidemiology and Infection* **124**(2): 207-213.
- Chapman, P. A., Siddons, C. A., Zadik, P. M., and Jewes, L.** (1991). An improved selective medium for the isolation of *Escherichia coli* O157. *Journal of Medical Microbiology* **35**(2): 107-110.
- Chart, H., Jenkins, C., Smith, H. R., Hedges, D., and Rowe, B.** (1998). Haemolysin production by strains of verocytotoxin-producing *Escherichia coli*. *Microbiology-Uk* **144**(Pt1): 103-107.
- Cheasty, T., Robertson, R., Chart, H., Mannion, P., Syed, Q., Garvey, R., and Rowe, B.** (1998). The use of serodiagnosis in the retrospective investigation of a nursery outbreak associated with *Escherichia coli* O157:H7. *Journal of Clinical Pathology* **51**(7): 498-501.

- China, B., Goffaux, F., Pirson, V., and Mainil, J.** (1999). Comparison of *eae*, *tir*, *EspA* and *EspB* genes of bovine and human attaching and effacing *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Microbiology Letters* **178**(1): 177-182.
- China, B., Jacquemin, E., Devrin, A. C., Pirson, V., and Mainil, J.** (1999). Heterogeneity of the *eae* genes in attaching effacing *Escherichia coli* from cattle: comparison with human strains. *Research in Microbiology* **150**(5): 323-332.
- Cobbold, R., and Desmarchelier, P.** (2000). A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC): prevalence in three Australian dairy herds. *Veterinary Microbiology* **71**(1-2): 125-137.
- Conlan, J.W., KuoLee, R., Webb, A., Cox, A.D. and 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 colonisation by the pathogen. *Canadian Journal of Microbiology* **46**(3): 283-290.
- Conlan, J.W. and Perry, M.B.** (1998). Susceptibility of three strains of conventional adult mice to intestinal colonisation by an isolate of *Escherichia coli* O157:H7. *Canadian Journal of Microbiology* **44**(8): 800-805.
- Cookson, A. L., and Woodward, M. J.** (2003). The role of intimin in the adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to HEp-2 tissue culture cells and to bovine gut explant tissues. *International Journal of Medical Microbiology* **292**(7-8): 547-553.
- Cooling, L. L. W., Walker, K. E., Gille, T., and Koerner, T. A. W.** (1998). Shiga toxin binds human platelets via globotriaosylceramide (P-k antigen) and a novel platelet glycosphingolipid. *Infection and Immunity* **66**(9): 4355-4366.
- Cornick, N. A., Booher, S. L., Casey, T. A., and Moon, H. W.** (2000). Persistent colonization of sheep by *Escherichia coli* O157:H7 and other *E. coli* pathotypes. *Applied and Environmental Microbiology* **66**.
- Cornick, N. A., Booher, S. L., and Moon, H. W.** (2002). Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. *Infection and Immunity* **70**(5): 2704-2707.
- Crane, J. K., McNamara, B. P., and Donnenberg, M. S.** (2001). Role of *EspF* in host cell death induced by enteropathogenic *Escherichia coli*. *Cellular Microbiology* **3**(4): 197-211.
- Cray Jr, W. C., and Moon, H. W.** (1995). Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* **61**: 1586-1590.

- Cray, W. C., and Moon, H. W.** (1995). Experimental-Infection of Calves and Adult Cattle With *Escherichia-Coli* O157:H7. *Applied and Environmental Microbiology* 61(4): 1586-1590.
- de Grado, M., Abe, A., Gauthier, A., Steele-Mortimer, O., DeVinney, R., and Finlay, B. B.** (1999). Identification of the intimin-binding domain of Tir of enteropathogenic *Escherichia coli*. *Cellular Microbiology* 1(1): 7-17.
- Dean-Nystrom, E. A., Bosworth, B. T., Cray, W. C., and Moon, H. W.** (1997). Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infection and Immunity* 65(5): 1842-1848.
- Dean-Nystrom, E. A., Bosworth, B. T., and Moon, H. W.** (1999a). Pathogenesis of *Escherichia coli* O157:H7 in weaned calves. *Advances in Experimental Medicine and Biology* 473: 173-177.
- Dean-Nystrom, E. A., Bosworth, B. T., Moon, H. W., and O'Brien, A. D.** (1998). *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. *Infection and Immunity* 66(9): 4560-4563.
- Dean-Nystrom, E. A., Bosworth, B. T., O'Brien, A. D., and Moon, H. W.** (1999b). Bovine Infection with *Escherichia coli* O157:H7. In *Escherichia coli* in Farm Animals, C. S. Stewart and H. J. Flint, eds. (Oxon: CABI Publishing), pp. 51-58.
- Dibb-Fuller, M. P., Best, A., Stagg, D. A., Cooley, W. A., and Woodward, M. J.** (2001). An *in vitro* model for studying the interaction of *Escherichia coli* O157:H7 and other enteropathogens with bovine primary cell cultures. *Journal of Medical Microbiology* 50(9): 759-769.
- Djafari, S., Ebel, F., Deibel, C., Kramer, S., Hdel, M., and Chakraborty, T.** (1997). Characterization of an exported protease from Shiga toxin-producing *Escherichia coli*. *Molecular Microbiology* 25(4): 771-784.
- Donnenberg, M. S., and Kaper, J. B.** (1991). Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* 59(12): 4310-4317.
- Donnenberg, M. S., Tacket, C. O., James, S. P., Losonsky, G., Nataro, J. P., Wasserman, S. S., Kaper, J. B., and Levine, M. M.** (1993). Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. *Journal of clinical investigation* 92(3): 1412-1417.
- Donnenberg, M. S., Tzipori, S., McKee, M. L., O'Brien, A. D., Alroy, J., and Kaper, J. B.** (1993). The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment *in vitro* and in a porcine model. *Journal of Clinical Investigation* 92(3): 1418-1424.

Doughty, S., Sloan, J., Bennett-Wood, V., Robertson, M., Robins-Browne, R. M., and Hartland, E. L. (2002). Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli*. *Infect Immun* **70**(12): 6761-6769.

Ebel, F., Deibel, C., Kresse, A. U., Guzman, C. A., and Chakraborty, T. (1996). Temperature- and medium-dependent secretion of proteins by Shiga toxin-producing *Escherichia coli*. *Infection and Immunity* **64**(11): 4472-4479.

Eisenhauer, P. B., Chaturvedi, P., Fine, E. R., Ritchie, A. J., Pober, J. S., Cleary, T. G., and Newburg, D. S. (2001). Tumor necrosis factor alpha increases human cerebral endothelial cell Gb₃ and sensitivity to Shiga toxin. *Infection and Immunity* **69**: 1889-1894.

Eisenstein, B. I. (1981). Phase Variation of Type-1 Fimbriae in *Escherichia coli* Is Under Transcriptional Control. *Science* **214**(4518): 337-339.

Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, G. A., Koohmaraie, M., and Laegreid, W. W. (2000). Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences of the United States of America* **97**(7): 2999-3003.

Elliott, S. J., Sperandio, V., Girón, J. A., Shin, S., Mellies, J. L., Wainwright, L., Hutcheson, S. W., McDaniel, T. K., and Kaper, J. B. (2000). The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE and non-LEE-encoded virulence factors in Enteropathogenic and Enterohemorrhagic *Escherichia coli*. *Infection and Immunity* **68**: 6115-6126.

Elliott, S. J., Yu, J., and Kaper, J. B. (1999). The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. *Infection and Immunity* **67**(8): 4260-4263.

Enami, M., Nakasone, N., Honma, Y., Kakinohana, S., Kudaka, J., and Iwanaga, M. (1999). Expression of type I pili is abolished in verotoxin-producing *Escherichia coli* O157. *Fems Microbiology Letters* **179**(2): 467-472.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T., and Igarashi, K. (1988). Site of Action of a Vero Toxin (Vt2) From *Escherichia-Coli* O157-H7 and of Shiga Toxin On Eukaryotic Ribosomes - RNA N- Glycosidase Activity of the Toxins. *European Journal of Biochemistry* **171**(1-2): 45-50.

Esco, M. A., Hazlett, L. D., and Kurpakus-Wheater, M. (2002). *Pseudomonas aeruginosa* binds to extracellular matrix deposited by human corneal epithelial cells. *Investigative Ophthalmology & Visual Science* **43**(12): 3654-3659.

- Fagan, P. K., Hornitzky, M. A., Bettelheim, K. A., and Djordjevic, S. P.** (1999). Detection of shiga-like toxin (stx(1) and stx(2)), intimin (eaeA), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. *Applied and Environmental Microbiology* **65**(2): 868-872.
- Faith, N. G., Shere, J. A., Brosch, R., Arnold, K. W., Ansay, S. E., Lee, M. S., and Luchansky, J. B. K., C. W.** (1996). Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology* **62**:1519-1525.
- FDA.** (1995). FDA Bacteriological Analytical Manual, 8th Edition (Arlington, VA: AOAC International).
- Ferreira, A. J., Elias, W. P., Jr., Pelayo, J. S., Giraldi, R., Pedroso, M. Z., and Scaletsky, I. C.** (1997). Culture supernatant of Shiga toxin-producing *Escherichia coli* strains provoke fluid accumulation in rabbit ileal loops. *FEMS Immunology and Medical Microbiology* **19**(4): 285-288.
- Finlay, B. B., and Falkow, S.** (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews* **61**(2): 136-169.
- Fischer, J. R., Zhao, T., Doyle, M. P., Goldberg, M. R., Brown, C. A., Sewell, C. T., Kavanaugh, D. M., and Bauman, C. D.** (2001). Experimental and field studies of *Escherichia coli* O157 : H7 in white-tailed deer. *Applied and Environmental Microbiology* **67**(3): 1218-1224.
- Fitzhenry, R. J., Pickard, D. J., E.L., H., Reece, S., Dougan, G., Phillips, A. D., and Frankel, G.** (2002). Intimin type influences the site of human intestinal mucosa colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. *Gut* **50**(2): 180-185.
- Fitzhenry, R. J., Reece, S., Trabulsi, L. R., Heuschkel, R., Murch, S., Thomson, A., Frankel, G., and Phillips, A. D.** (2002). Tissue tropism of enteropathogenic *Escherichia coli* strains belonging to the O55 serogroup. *Infection and Immunity* **70**(8): 4362-4368.
- Foster, G. H., Armstrong, C. S., Sakiri, R., and Tesh, V. L.** (2000). Shiga toxin-induced tumor necrosis factor alpha expression: Requirement for toxin enzymatic activity and monocyte protein kinase C and protein tyrosine kinases. *Infection and Immunity* **68**: 5183-5189.
- Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B., and Knutton, S.** (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology* **30**(5): 911-921.
- Frost, J. A., Cheasty, T., Thomas, A., and Rowe, B.** (1993). Phage typing of vero cytotoxin-producing *Escherichia-Coli* O-157 Isolated in the United-Kingdom - 1989-91. *Epidemiology and Infection* **110**(3): 469-475.

Fuchs, S., Muhldorfer, I., DonohueRolfé, A., Kerenyi, M., Emody, L., Alexiev, R., Nenkov, P., and Hacker, J. (1999). Influence of RecA on *in vivo* virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. *Microbial Pathogenesis* 27(1): 13-23.

Fukushima, H., Hashizume, T., Morita, Y., Tanaka, J., Azuma, K., Mizumoto, Y., Kaneno, M., Matsuura, M. O., Konma, K., and Kitani, T. (1999). Clinical experiences in Sakai City Hospital during the massive outbreak of enterohemorrhagic *Escherichia coli* O157 infections in Sakai City, 1996. *Pediatrics International* 41(2): 213-217.

Gautom, R. K. (1997). Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. *Journal of Clinical Microbiology* 35(11): 2977-2980.

Gilsdorf, J. R., McCrea, K. W., and Marrs, C. F. (1997). Role of pili in *Haemophilus influenzae* adherence and colonization. *Infection and Immunity* 65(8): 2997-3002.

Giron, J. A., Ho, A. S. Y., and Schoolnik, G. K. (1991). An Inducible Bundle-Forming Pilus of Enteropathogenic *Escherichia-Coli*. *Science* 254(5032): 710-713.

Grauke, L. J., Kudva, I. T., Yoon, J. W., Hunt, C. W., Williams, C. J., and Hovde, C. J. (2002). Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. *Applied and Environmental Microbiology* 68(5): 2269-2277.

Gunning, R. F., Wales, A. D., Pearson, G. R., Done, E., Cookson, A. L., and Woodward, M. J. (2001). Attaching and effacing lesions in the intestines of two calves associated with natural infection with *Escherichia coli* O26:H11. *Veterinary Record* 148(25): 780-782.

Gunzer, F., Hennig-Pauka, I., Waldmann, K. H., Sandhoff, R., Grone, H. J., Kreipe, H. H., Matussek, A., and Mengel, M. (2002). Gnotobiotic piglets develop thrombotic microangiopathy after oral infection with enterohemorrhagic *Escherichia coli*. *American Journal of Clinical Pathology* 118(3): 364-375.

Hacker, J., Kestler, H., Hoschutzky, H., Jann, K., Lottspeich, F., and Korhonen, T. K. (1993). Cloning and Characterization of the S-Fimbrial Adhesin-Ii Complex of an *Escherichia coli* O18:K1 Meningitis Isolate. *Infection and Immunity* 61(2): 544-550.

Hall, G. A., Reynolds, D. J., Chanter, N., Morgan, J. H., Parsons, K. R., Debney, T. G., Bland, A. P., and Bridger, J. C. (1985). Dysentery Caused By *Escherichia coli* (S102-9) in Calves - Natural and Experimental Disease. *Veterinary Pathology* 22(2): 156-163.

- Hancock, D. D., Besser, T. E., Gill, C., and Bohach, C. H.** (1999). Cattle, hay, and *E. coli*. *Science* **284**(5411): 51-52.
- Hancock, D. D., Rice, D. H., Herriott, D. E., Besser, T. E., Ebel, E. D., and Carpenter, L. V.** (1997). Effects of farm manure-handling practices on *Escherichia coli* O157 prevalence in cattle. *Journal of Food Protection* **60**(4): 363-366.
- Hancock, I., and Poxton, I.** (1988). *Bacterial Cell Surface Techniques* (Chichester: Wiley).
- Hanski, E., and Caparon, M.** (1992). Protein-F, a Fibronectin-Binding Protein, Is an Adhesin of the Group-A Streptococcus *Streptococcus pyogenes*. *Proceedings of the National Academy of Sciences of the United States of America* **89**(13): 6172-6176.
- Harmon, B. G., Brown, C. A., Tkalcic, S., Mueller, P. O. E., Parks, A., Jain, A. V., Zhao, T., and Doyle, M. P.** (1999). Fecal shedding and rumen growth of *Escherichia coli* O157:H7 in fasted calves. *Journal of Food Protection* **62**(6): 574-579.
- Hartland, E. L., Batchelor, M., Delahay, R. M., Hale, C., Matthews, S., Dougan, G., Knutton, S., Connerton, I., and Frankel, G.** (1999). Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Molecular Microbiology* **32**(1): 151-158.
- Hartland, E. L., Huter, V., Higgins, L. M., Goncalves, N. S., Dougan, G., Phillips, A. D., MacDonald, T. T., and Frankel, G.** (2000). Expression of Intimin γ from Enterohemorrhagic *Escherichia coli* in *Citrobacter rodentium*. *Infection and Immunity* **68**: 4637-4646.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E., and Holden, D. W.** (1995). Simultaneous Identification of Bacterial Virulence Genes By Negative Selection. *Science* **269**(5222): 400-403.
- Hicks, S., Candy, D. C. A., and Phillips, A. D.** (1996). Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. *Infection and Immunity* **64**(11): 4751-4760.
- Hicks, S., Frankel, G., Kaper, J. B., Dougan, G., and Phillips, A. D.** (1998). Role of intimin and bundle-forming pili in enteropathogenic *Escherichia coli* adhesion to pediatric intestinal tissue in vitro. *Infection and Immunity* **66**(4): 1570-1578.
- Hoey, D. E., Currie, C., Else, R. W., Nutikka, A., Lingwood, C. A., Gally, D. L., and Smith, D. G. E.** (2002). Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. *Journal of Medical Microbiology* **51**: 143-149.

Hoey, D. E. E., Sharp, L., Currie, C., Lingwood, C. A., Gally, D. L., and Smith, D. G. E. (2003). Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. *Cellular Microbiology* **5**(2): 85-97.

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Review of Nutrition* **22**: 283-307.

Hovde, C. J., Austin, P. R., Cloud, K. A., Williams, C. J., and Hunt, C. W. (1999). Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Applied and Environmental Microbiology* **65**(7): 3233-3235.

Hueck, J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews* **62**(2): 379-433.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L., and Boren, T. (1998). *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279**(5349): 373-377.

Isberg, R. R., and Leong, J. M. (1990). Multiple Beta-1-Chain Integrins Are Receptors For Invasin, a Protein That Promotes Bacterial Penetration Into Mammalian- Cells. *Cell* **60**(5): 861-871.

Jacewicz, M. S., Acheson, D. W. K., Binion, D. G., West, G. A., Lincicome, L. L., Fiocchi, C., and Keusch, G. T. (1999). Responses of human intestinal microvascular endothelial cells to shiga toxins 1 and 2 and pathogenesis of hemorrhagic colitis. *Infection and Immunity* **67**(3): 1439-1444.

Jackson, M. P., Neill, R. J., O'Brien, A. D., Holmes, R. K., and Newland, J. W. (1987). Nucleotide-Sequence Analysis and Comparison of the Structural Genes For Shiga-Like Toxin-I and Shiga-Like Toxin-Ii Encoded By Bacteriophages From *Escherichia coli* 933. *FEMS Microbiology Letters* **44**(1): 109-114.

James, C. E., Stanley, K. N., Allison, H. E., Flint, H. J., Stewart, C. S., Sharp, R. J., Saunders, J. R., and McCarthy, A. J. (2001). Lytic and lysogenic infection of diverse *Escherichia coli* and *Shigella* strains with a verocytotoxigenic bacteriophage. *Applied and Environmental Microbiology* **67**(9): 4335-4337.

Jenkins, C., Chart, H., Cheasty, T., Willshaw, G. A., Pearce, M. C., Foster, G., Gunn, G. J., Smith, H. R., Dougan, G., Synge, B. A., and Frankel, G. (2002). Verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 from Scottish cattle. *Veterinary Record* **151**(2): 58-60.

Jenkins, C., Pearce, M. C., Chart, H., Cheasty, T., Willshaw, G. A., Gunn, G. J., Dougan, G., Smith, H. R., Synge, B. A., and Frankel, G. (2002). An eight-month

study of a population of verocytotoxigenic *Escherichia coli* (VTEC) in a Scottish cattle herd. *Journal of Applied Microbiology* **93**(6): 944-53.

Jensen, V. B., Harty, J. T., and Jones, B. D. (1998). Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. *Infection and Immunity* **66**(8): 3758-3766.

Jerse, A. E., Yu, J., Tall, B. D., and J.B., K. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 7839-7843.

Johnson, L. A., and Boedeker, E. C. (2000). Evaluation of antibiotic therapy in a rabbit model of shiga toxin-producing *E. coli* (STEC) infection: No evidence for adverse effects. *Gastroenterology* **118**(4): 1760.

Kallstrom, H., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Molecular Microbiology* **25**(4): 639-647.

Karch, H., Heesemann, J., Laufs, R., O'Brien, A. D., Tacket, C. O., and Levine, M. M. (1987). A plasmid of enterohaemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. *Infection and Immunity* **55**(2): 455-461.

Karch, H., Janetzki, Mittmann, C., Aleksic, S., and Datz, M. (1996). Isolation of enterohemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. *Journal of Clinical Microbiology* **34**(3): 516-519.

Karmali, M. A., Petric, M., Steele, B. T., and Lim, C. (1983). Sporadic Cases of Hemolytic-Uremic Syndrome Associated With Fecal Cyto-Toxin and Cytotoxin-Producing *Escherichia coli* in Stools. *Lancet* **1**(8325): 619-620.

Karpman, D., Connell, H., Svensson, M., Scheutz, F., Alm, P. and Svenborg, C. (1997). The role of lipopolysaccharide and Shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. *Journal of Infectious Disease* **175**(3): 611-620.

Kelly, G., Prasannan, S., Daniell, S., Fleming, K., Frankel, G., Dougan, G., Connerton, I., and Matthews, S. (1999). Structure of the cell-adhesion fragment of intimin from enteropathogenic *Escherichia coli*. *Nature Structural Biology* **6**(4): 313-318.

Kenny, B. (2001). The enterohaemorrhagic *Escherichia coli* (serotype O157:H7) Tir molecule is not functionally interchangeable for its enteropathogenic *E-coli* (serotype O127 : H6) homologue. *Cellular Microbiology* **3**(8): 499-510.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., and Finlay, B. B. (1997). Enteropathogenic *E-coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**(4): 511-520.

Kenny, B., Ellis, S., Leard, A. D., Warawa, J., Mellor, H., and Jepson, M. A. (2002). Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. *Molecular Microbiology* **44**(4): 1095-1107.

Kenny, B., and Warawa, J. (2001). Enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule does not undergo full modification when introduced into host cells by EPEC-independent mechanisms. *Infection and Immunity* **69**(3): 1444-1453.

Knutton, S., Baldwin, T., Williams, P. H., and McNeish, A. S. (1989). Actin Accumulation At Sites of Bacterial Adhesion to Tissue- Culture Cells - Basis of a New Diagnostic-Test For Enteropathogenic and Enterohemorrhagic *Escherichia coli*. *Infection and Immunity* **57**(4): 1290-1298.

Konowalchuck, J., Speirs, J. I., and Stavric, S. (1977). Vero response to a cytotoxin of *Escherichia. coli*. *Infection and Infection* **18**: 775-779.

Kudva, I. T., Hatfield, P. G., and Hovde, C. J. (1995). Effect of Diet On the Shedding of *Escherichia-Coli* O157-H7 in a Sheep Model. *Applied and Environmental Microbiology* **61**(4): 1363-1370.

Kudva, I. T., Hatfield, P. G., and Hovde, C. J. (1996). *Escherichia coli* O157:H7 in microbial flora of sheep. *Journal of Clinical Microbiology* **34**(2): 431-433.

Laegreid, W. W., Elder, R. O., and Keen, J. E. (1999). Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiology and Infection* **123**(2): 291-298.

Li, Y. L., Frey, E., MacKenzie, A. M. R., and Finlay, B. B. (2000). Human response to *Escherichia coli* O157:H7 infection: Antibodies to secreted virulence factors. *Infection and Immunity* **68**(9): 5090-5095.

Licence, K., Oates, K. R., Syngé, B. A., and Reid, T. M. (2001). An outbreak of *E. coli* O157 infection with evidence of spread from animals to man through contamination of a private water supply. *Epidemiology and Infection* **126**(1): 135-138.

Liebler, E. M., Pohlenz, J. F., and Woode, G. N. (1988). Gut-Associated Lymphoid-Tissue in the Large-Intestine of Calves .1. Distribution and Histology. *Veterinary Pathology* **25**(6): 503-508.

Lindgren, S.W., Melton, A.R. and O'Brien, A.D. (1993). Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model. *Infection and Immunity* **61**(9): 3832-3842.

- Lingwood, C. A.** (1996). Role of verotoxin receptors in pathogenesis. *Trends in Microbiology* 4(4): 147-153.
- Lissner, R., Schmidt, H. and Karch H.** (1996). A standard immunoglobulin preparation produced from bovine colostrum shows antibody reactivity and neutralization activity against Shiga-like toxins and EHEC hemolysin of *Escherichia coli* O157:H7. *Infection* 24(5): 378-383.
- Louie, M., Read, S., Louie, L., Ziebell, K., Rahn, K., Borczyk, A., and Lior, H.** (1999). Molecular typing methods to investigate transmission of *Escherichia coli* O157:H7 from cattle to humans. *Epidemiology and Infection* 123(1): 17-24.
- Ludwig, K., Sarkim, V., Bitzan, M., Karmali, M. A., Bobrowski, C., Ruder, H., Laufs, R., Sobottka, I., Petric, M., Karch, H., and Muller-Wiefel, D. E.** (2002). Shiga toxin-producing *Escherichia coli* infection and antibodies against Stx2 and Stx1 in household contacts of children with enteropathic hemolytic-uremic syndrome. *Journal of Clinical Microbiology* 40(5): 1773-1782.
- Luo, Y., Frey, E. A., Pfuetzner, R. A., Creagh, A. C., Knoechel, D. G., Haynes, C. A., Finlay, B. B., and Strynadka, N. C.** (2000). Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature* 405: 1073-1077.
- Madico, G., Akopyants, N. S., and Berg, D. E.** (1995). Arbitrarily Primed PCR DNA-Fingerprinting of *Escherichia-Coli* O157:H7 Strains By Using Templates From Boiled Cultures. *Journal of Clinical Microbiology* 33(6): 1534-1536.
- Magnuson, B. A., Davis, M., Hubele, S., Austin, P. R., Kudva, I. T., Williams, C. J., Hunt, C. W., and Hovde, C. J.** (2000). Ruminant gastrointestinal cell proliferation and clearance of *Escherichia coli* O157 : H7. *Infection and Immunity* 68(7): 3808-3814.
- March, S. B., and Ratnam, S.** (1986). Sorbitol-MacConkey Medium For Detection of *Escherichia coli* O157:H7 Associated With Hemorrhagic Colitis. *Journal of Clinical Microbiology* 23(5): 869-872.
- Martin, I. E., Tyler, S. D., Tyler, K. D., Khakhria, R., and Johnson, W. M.** (1996). Evaluation of ribotyping as epidemiologic tool for typing *Escherichia coli* serogroup O157 isolates. *Journal of Clinical Microbiology* 34(3): 720-723.
- Matise, I., Cornick, N. A., Booher, S. L., Samuel, J. E., Bosworth, B. T., and Moon, H. W.** (2001). Intervention with shiga toxin (Stx) antibody after infection by Stx-producing *Escherichia coli*. *Journal of Infectious Diseases* 183(2): 347-350.
- Matsushiro, A., Sato, K., Miyamoto, H., Yamamura, T., and Honda, T.** (1999). Induction of prophages of enterohemorrhagic *Escherichia coli* O157:H7 with norfloxacin. *Journal of Bacteriology* 181(7): 2257-2260.

- McCullagh, P., and Nelder, J. A.** (1989). *Generalised Linear Models*, 2nd Edition (London: Chapman and Hall).
- McDaniel, T. K., and Kaper, J. B.** (1997). A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K12. *Molecular Microbiology* **23**(2): 399-407.
- McKee, M. L., Meltoncelsa, A. R., Moxley, R. A., Francis, D. H., and O'Brien, A. D.** (1995). Enterohemorrhagic *Escherichia coli* O157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to Hep 2 cells. *Infection and Immunity* **63**(9): 3739-3744.
- McNally, A., Roe, A. J., Simpson, S., Thomson-Carter, F. M., Hoey, D. E. E., Currie, C., Chakraborty, T., Smith, D. G. E., and Gally, D. L.** (2001). Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. *Infection and Immunity* **69**(8): 5107-5114.
- Mead, R.** (1990). *The Design of Experiments* (Cambridge: Cambridge University Press), pp. 150-154.
- MeyerRosberg, K., and Berglindh, T.** (1996). *Helicobacter* colonization of biopsy specimens cultured in vitro is dependent on both mucosal type and bacterial strain. *Scandinavian Journal of Gastroenterology* **31**(5): 434-441.
- Meylan, M., Eicher, R., Blum, J. W., and Steiner, A.** (2002). Effects of an abrupt increase of starch-rich concentrates in the diet of dairy cows on concentrations of volatile fatty acids in the rumen and large intestine and on myoelectric activity of the spiral colon. *American Journal of Veterinary Research* **63**(6): 857-867.
- Middleton, A. M., Dowling, R. B., Mitchell, J. L., Watanabe, S., Rutman, A., Pritchard, K., Tillotson, G., Hill, S. L., and Wilson, R.** (2003). Haemophilus parainfluenzae infection of respiratory mucosa. *Respiratory Medicine* **97**(4): 375-381.
- Miyao, Y., Kataoka, T., Nomoto, T., Kai, A., Itoh, T., and Itoh, K.** (1998). Prevalence of verotoxin-producing *Escherichia coli* harbored in the intestine of cattle in Japan. *Veterinary Microbiology* **61**(1-2): 137-143.
- Moon, H. W., Isaacson, R. E., and Pohlenz, J.** (1979). Mechanisms of association of enteropathogenic *Escherichia coli* with intestinal epithelium. *American Journal of Clinical Nutrition* **32**(1): 119-127.
- Mouricout, M.** (1991). Swine and Cattle Enterotoxigenic *Escherichia-Coli*-Mediated Diarrhea - Development of Therapies Based On Inhibition of Bacteria-Host Interactions. *European Journal of Epidemiology* **7**(6): 588-604.

Moxley, R. A., and Francis, D. H. (1986). Natural and Experimental-Infection With an Attaching and Effacing Strain of *Escherichia-Coli* in Calves. *Infection and Immunity* **53**(2): 339-346.

Mukherjee, J., Chios, K., Fishwild, D., Hudson, D., O'Donnell, S., Rich, S.M., Donohue-Rolfe, A. and Tzipori, S. (2002). Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. *Infection and Immunity* **70**(2): 612-619.

Nagano, K., Taguchi, K., Hara, T., Yokoyama, S., Kawada, K. and Mori, H. (2003). Adhesion and colonization of enterohemorrhagic *Escherichia coli* O157:H7 in cecum of mice . *Microbiology and Immunology* **47**(2):125-132.

Nagy, B., and Fekete, P. Z. (1999). Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. *Veterinary Research* **30**(2-3): 259-284.

Nakazawa, M., Akiba, M., and Sameshima, T. (1999). Swine as a potential reservoir of Shiga toxin-producing *Escherichia coli* O157:H7 in Japan. *Emerging Infectious Diseases* **5**(6): 833-834.

Nataro, J. P., and Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews* **11**(1): 142-201.

Neidhardt, F. C. (1996). The enteric bacterial cell and the age of bacteria. In *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, F. C. Neidhardt, ed. (Washington DC: ASM Press), pp. 1-3.

Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974). Culture media for enterobacteria. *Journal of Bacteriology* **119**: 736-747.

Nicholls, L., Grant, T. H., and RobinsBrowne, 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. *Molecular Microbiology* **35**(2): 275-288.

Nielsen, E. M., Tegtmeier, C., Andersen, H. J., Gronbaek, C., and Andersen, J. S. (2002). Influence of age, sex and herd characteristics on the occurrence of Verocytotoxin-producing *Escherichia coli* O157 in Danish dairy farms. *Veterinary Microbiology* **88**(3): 245-257.

O'Brien, A. D., and Holmes, R. K. (1987). Shiga and Shiga-Like Toxins. *Microbiological Reviews* **51**(2): 206-220.

O'Brien, A. D., Newland, J. W., Miller, S. F., Holmes, R. K., Smith, H. W., and Formal, S. B. (1984). Shiga-Like Toxin-Converting Phages From *Escherichia coli* Strains That Cause Hemorrhagic Colitis or Infantile Diarrhea. *Science* **226**(4675): 694-696.

- Ofek, I., Mirelman, D., and Sharon, N.** (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature* **265**: 623-625.
- Ogawa, M., Shimizu, K., Nomoto, K., Takahashi, M., Watanuki, M., Tanaka, R., Tanaka, T., Hamabata, T., Yamasaki, S., and Takeda, Y.** (2001). Protective effect of *Lactobacillus casei* strain Shirota on Shiga toxin-producing *Escherichia coli* O157:H7 infection in infant rabbits. *Infect Immun* **69**(2): 1101-1108.
- Ogden, I. D., Hepburn, N. F., MacRae, M., Strachan, N. J. C., Fenlon, D. R., Rusbridge, S. M., and Pennington, T. H.** (2002). Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Letters in Applied Microbiology* **34**(2): 100-104.
- Ohmi, K., Kiyokawa, N., Takeda, T., and Fujimoto, J.** (1998). Human microvascular endothelial cells are strongly sensitive to Shiga toxins. *Biochemical and Biophysical Research Communications* **251**(1): 137-141.
- Ohya, T., Marubashi, T., and 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. *Journal of Veterinary Medical Science* **62**(11): 1151-1155.
- Okrend, A. J. G., Rose, B. E., and Bennett, B.** (1990). A Screening Method For the Isolation of *Escherichia-Coli* O157- H7 From Ground-Beef. *Journal of Food Protection* **53**(3): 249-252.
- Orskov, F., Orskov, I., and Villar, J. A.** (1987). Cattle as a reservoir of verotoxin-producing *Escherichia coli* O157:H7. *The Lancet* **2(8553)**: 276-276.
- Ostroff, S. M., Griffin, P. M., Tauxe, R. V., Shipman, L. D., Greene, K. D., Wells, J. G., Lewis, J. H., Blake, P. A., and Kobayashi, J. M.** (1990). A statewide outbreak of *Escherichia coli* O157:H7 infections in Washington State. *American Journal of Epidemiology* **132**: 239-247.
- Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marches, O., and Caprioli, A.** (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: Characterization of a new intimin variant. *Infection and Immunity* **68**(1): 64-71.
- Padhye, N. V., and Doyle, M. P.** (1991). Rapid Procedure For Detecting Enterohemorrhagic *Escherichia- Coli* O157:H7 in Food. *Applied and Environmental Microbiology* **57**(9): 2693-2698.
- Paiba, G. A., Gibbens, J. C., Pascoe, S. J. S., Wilesmith, J. W., Kidd, S. A., Byrne, C., Ryan, J. B. M., Smith, R. R., McLaren, I. M., Futter, R. J., Kay, A. C. S., Jones, Y. E., Chappell, S. A., Willshaw, G. A., and Cheasty, T.** (2002). Faecal carriage of verocytotoxin-producing *Escherichia coli* O157 in cattle and sheep at slaughter in Great Britain. *Veterinary Record* **150**(19): 593-598.

- Pan, Y. T., xu, B., Rice, K., Smith, S., Jackson, R., and Elbein, A. D.** (1997). Specificity of the high-mannose recognition site between *Enterobacter cloacae* pili adhesin and HT-29 cell membranes. *Infection and Immunity* **65**(10): 4199-4206.
- Park, C. H., Vandell, N. M., and Hixon, D. L.** (1996). Rapid immunoassay for detection of *Escherichia coli* O157 directly from stool specimens. *Journal of Clinical Microbiology* **34**(4): 988-990.
- Paros, M., Tarr, P. I., Kim, H., Besser, T. E., and Hancock, D. D.** (1993). A comparison of human and bovine *Escherichia coli* O157:H7 isolates by toxin genotype, plasmid profile, and bacteriophage lambda-restriction fragment length polymorphism profile. *Journal of Infectious Diseases* **168**(5): 1300-1303.
- Paton, A. W., Morona, R., and Paton, J. C.** (2001). Neutralization of shiga toxins Stx1, Stx2c, and Stx2e by recombinant bacteria expressing mimics of globotriose and globotetraose. *Infection and Immunity* **69**(3): 1967-1970.
- Pearson, G. R., Bazeley, K. J., Jones, J. R., Gunning, R. F., Green, M. J., Cookson, A., and 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. *Veterinary Record* **145**(13): 370-373.
- Pearson, G. R., Watson, C. A., Hall, G. A., and Wray, C.** (1989). Natural infection with an attaching and effacing *Escherichia coli* in the small and large intestines of a calf with diarrhoea. *The Veterinary Record* **124**(12): 297-299.
- Perna, N. T., Plunkett, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Limk, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J. Y., Yen, G., Schwartz, D. C., Welch, R. A., and Blattner, F. R.** (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**(6819): 529-533.
- Phillips, A. D., and Frankel, G.** (2000). Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with woman intestinal organ cultures. *Journal of Infectious Diseases* **181**(4): 1496-1500.
- Phillips, A. D., Giron, J., Hicks, S., Dougan, G., and Frankel, G.** (2000). Intimin from enteropathogenic *Escherichia coli* mediates remodelling of the eukaryotic cell surface. *Microbiology-Uk* **146**: 1333-1344.
- Phillips, A. D., Navabpour, S., Hicks, S., Dougan, G., Wallis, T., and Frankel, G.** (2000). Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* **47**(3): 377-381.

- Philpott, D. J., Ackerley, C. A., Kiliaan, A. J., Karmali, M. A., Perdue, M. H., and Sherman, P. M.** (1997). Translocation of verotoxin-1 across T84 monolayers: mechanism of bacterial toxin penetration of epithelium. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **36**(6): G1349-G1358.
- Pickering, L. K., Obrig, T. G., and Stapleton, F. B.** (1994). Hemolytic-Uremic Syndrome and Enterohemorrhagic *Escherichia coli*. *Pediatric Infectious Disease Journal* **13**(6): 459-475.
- Porter, E. M., Bevins, C. L., Ghosh, D., and Ganz, T.** (2002). The multifaceted Paneth cell. *Cellular and Molecular Life Sciences* **59**(1): 156-170.
- Posfai, G., Kolisnychenko, V., Berezki, Z., and Blattner, F. R.** (1999). Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucleic Acids Research* **27**(22): 4409-4415.
- Posfai, G., Koob, M. D., Kirkpatrick, H. A., and Blattner, F. R.** (1997). Versatile insertion plasmids for targeted genome manipulations in bacteria: Isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* O157:H7 genome. *Journal of Bacteriology* **179**(13): 4426-4428.
- Potter, M. E., Kaufmann, A. F., Thomason, B. M., Blake, P. A., and Farmer, J. J.** (1985). Diarrhoea due to *Escherichia coli* O157:H7 in the infant rabbit. *Journal of infectious diseases* **152**(6): 1341-1343.
- Pritchard, G. C., Williamson, S., Carson, T., Bailey, J. R., Warner, L., Willshaw, G., and Cheasty, T.** (2001). Wild rabbits-a novel vector for verocytotoxigenic *Escherichia coli* O157. *Veterinary Record* **149**(18): 567-567.
- Proulx, F., Turgeon, J. P., Litalien, C., Mariscalco, M. M., Robitaille, P., and Seidman, E.** (1998). Inflammatory mediators in *Escherichia coli* O157:H7 hemorrhagic colitis and hemolytic-uremic syndrome. *Pediatric Infectious Disease Journal* **17**(10): 899-904.
- Reid, C. A., Avery, S. M., Warriss, P., and Buncic, S.** (2002). The effect of feed withdrawal on *Escherichia coli* shedding in beef cattle. *Food Control* **13**(6-7): 393-398.
- Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K., and Whittam, T. S.** (2000). Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* **406**(6791): 64-67.
- Richardson, S. E., Karmali, M. A., Becker, L. E., and Smith, C. R.** (1988). The Histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. *Human Pathology* **19**(9): 1102-1108.

- Richardson, S. E., Rotman, T. A., Jay, V., Smith, C. R., Becker, L. E., Petric, M., Olivieri, N. F., and Karmali, M. A.** (1992). Experimental Verocytotoxemia in Rabbits. *Infection and Immunity* **60**(10): 4154-4167.
- Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargrett, N. T., Blake, P. A., and Cohen, M. L.** (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* **308**(12): 681-685.
- Roberts, D.** (1995). *Practical Food Microbiology*, D. Roberts, W. H. Hooper and M. Greenwood, eds.: Public Health Laboratory Service).
- Roe, A. J., Currie, C., Smith, D. G. E., and Gally, D. L.** (2001). Analysis of type 1 fimbriae expression in verotoxigenic *Escherichia coli*: a comparison between serotypes O157 and O26. *Microbiology* **147**: 145-152.
- Ruggenti, P., and Remuzzi, G.** (1998). Pathophysiology and management of thrombotic microangiopathies. *Journal of Nephrology* **11**(6): 300-310.
- Sambrook, S., Fritsch, E. F., and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edition, C. Nolan, ed. (New York: Cold Spring Harbour Laboratory Press).
- Sanchez-SanMartin, C., Bustamante, V. W., Calva, E., and Puente, J. L.** (2001). Transcriptional regulation of the orf19 gene and the tir-cesT- eae operon of enteropathogenic *Escherichia coli*. *Journal of Bacteriology* **183**(9): 2823-2833.
- Sanderson, M. W., Besser, T. E., Gay, J. M., Gay, C. C., and Hancock, D. D.** (1999). Fecal *Escherichia coli* O157:H7 shedding patterns of orally inoculated calves. *Veterinary Microbiology* **69**(3): 199-205.
- Sanderson, M. W., Gay, J. M., Hancock, D. D., Gay, C. C., Fox, L. K., and Besser, T. E.** (1995). Sensitivity of Bacteriological Culture For Detection of *Escherichia coli* O157:H7 in Bovine Feces. *Journal of Clinical Microbiology* **33**(10): 2616-2619.
- Sandhu, K. S., and Gyles, C. L.** (2002). Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. *Canadian Journal of Veterinary Research- Revue Canadienne De Recherche Veterinaire* **66**(2): 65-72.
- Sandvig, K., Garred, O., Prydz, K., Kozlov, J. V., Hansen, S. H., and Vandeurs, B.** (1992). Retrograde Transport of Endocytosed Shiga Toxin to the Endoplasmic-Reticulum. *Nature* **358**(6386): 510-512.
- Sasaki, T., Kobayashi, M., and Agui, N.** (2000). Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera:Muscidae) in the dissemination of *Escherichia coli* O157:H7 to food. *Journal of Medical Entomology* **37**(6): 945-949.

Sata, S., Fujisawa, T., Osawa, R., Iguchi, A., Yamai, S., and Shimada, T. (2003). An improved enrichment broth for isolation of *Escherichia coli* O157, with specific reference to starved cells, from radish sprouts. *Applied and Environmental Microbiology* **69**(3): 1858-1860.

Savarino, S. J., Fasano, A., Watson, J., Martin, B. M., Levine, M. M., Guandalini, S., and Guerry, P. (1993). Enteroaggregative *Escherichia coli* heat-stable enterotoxin-1 represents another subfamily of *Escherichia coli* heat-stable toxin. *Proceedings of the National Academy of Sciences of the United States of America* **90**(7): 3093-3097.

Savarino, S. J., McVeigh, A., Watson, J., Cravioto, A., Molina, J., Echeverria, P., Bhan, M. K., Levine, M. M., and Fasano, A. (1996). Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. *Journal of Infectious Diseases* **173**(4): 1019-1022.

Schmidt, H., Beutin, L. and Karch, H. (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL933. *Infection and Immunity* **63**(3): 1055-1061.

Schmidt, H. and Karch, H. (1996). Enterohemolytic phenotypes and genotypes of shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhoea and hemolytic-uremic syndrome. *Journal of Clinical Microbiology* **34**(10): 2364-2367.

Schmidt, H., Kembach, C. and Karch, H. (1996). Analysis of the EHEC hly operon and its location in the physical map of the large plasmid of enterohemorrhagic *Escherichia coli* O157:H7. *Microbiology* **142**(4): 907-914.

Schmidt, H., Scheef, J., Morabito, S., Caprioli, A., Wieler, L. H., and Karch, H. (2000). A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Applied and Environmental Microbiology* **66**(3): 1205-1208.

Schoeni, J. L., and Doyle, M. P. (1994). Variable colonization of chickens perorally inoculated with *Escherichia coli* O157:H7 and subsequent contamination of eggs. *Applied and Environmental Microbiology* **60**(8): 2958-2962.

Schoonderwoerd, M., Clarke, R. C., Vandreuvel, A. A., and Rawluk, S. A. (1988). Colitis in Calves - Natural and Experimental-Infection With a Verotoxin-Producing Strain of *Escherichia coli* O111:NM. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* **52**(4): 484-487.

Scotland, S. M., Smith, H. R., Willshaw, G. A., and Rowe, B. (1983). Vero Cytotoxin Production in Strain of *Escherichia-Coli* Is Determined By Genes Carried On Bacteriophage. *Lancet* **2**(8343): 216-216.

Sears, C. L., and Kaper, J. B. (1996). Enteric bacterial toxins: Mechanisms of action and linkage to intestinal secretion. *Microbiological Reviews* **60**(1): 167-217.

- Sedgmen, B. J., Lofthouse, S. A., Scheerlinck, J. P. Y., and Meeusen, E. N. T.** (2002). Cellular and molecular characterisation of the ovine rectal mucosal environment. *Veterinary Immunology and Immunopathology* **86**(3-4): 215-220.
- Sheoran, A.S., Chapman, S., Singh, P., Donohue-Rolfe, A. and Tzipori, S.** (2003) Stx2-specific human monoclonal antibodies protect mice against lethal infection with *Escherichia coli* expressing Stx2 variants. *Infection and Immunity* **71**(6): 3125-3130.
- Shere, J. A., Kaspar, C. W., Bartlett, K. J., Linden, S. E., Norell, B., Francey, S., and Schaefer, D. M.** (2002). Shedding of *Escherichia coli* O157:H7 in dairy cattle housed in a confined environment following waterborne inoculation. *Applied and Environmental Microbiology* **68**(4): 1947-1954.
- Sinclair, J. F., and O'Brien, A. D.** (2002). Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. *Journal of Biological Chemistry* **277**(4): 2876-2885.
- Sjogren, R., Neill, R., Newland, J., Gemski, P., Mitcheltree, L., Pintado, I., and Boedeker, E.** (1987). Influence of Shiga-Like Toxin Production in Enteric Infection With an Enteropathogenic *Escherichia coli* Strain. *Gastroenterology* **92**(5): 1643-1643.
- Sowers, E. G., Wells, J. G., and Strockbine, N. A.** (1996). Evaluation of commercial latex reagents for identification of O157 and H7 antigens of *Escherichia coli*. *Journal of Clinical Microbiology* **34**(5): 1286-1289.
- Stapleton, A. E., Stroud, M. R., Hakomori, S. I., and Stamm, W. E.** (1998). The globoseries glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor *in vitro* for uropathogenic *Escherichia coli* expressing PAP-encoded adhesins. *Infection and Immunity* **66**(8): 3856-3861.
- Stevens, M. P., Marches, O., Campbell, J., Huter, V., Frankel, G., Phillips, A. D., Oswald, E., and Wallis, T. S.** (2002). Intimin, tir, and Shiga toxin 1 do not influence enteropathogenic responses to Shiga toxin-producing *Escherichia coli* in bovine ligated intestinal loops. *Infection and Immunity* **70**(2): 945-952.
- Stevens, M. P., van Diemen, P. M., Frankel, G., Phillips, A. D., and Wallis, T. S.** (2002). Efa1 influences colonization of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infection and Immunity* **70**(9): 5158-5166.
- Stordeur, P., China, B., Charlier, G., Roels, S., and Mainil, J.** (2000). Clinical signs, reproduction of attaching/effacing lesions, and enterocyte invasion after oral inoculation of an O118 enterohaemorrhagic *Escherichia coli* in neonatal calves. *Microbes and Infection* **2**(1): 17-24.

- Sussman, M., and Gally, D. L.** (1999). The Biology of Cystitis: host and bacterial factors. *Annual Reviews of Medicine* **50**: 149-158.
- Synge, B., and Paiba, C.** (2000). Verocytotoxin-producing *E coli* O157. *Veterinary Record* **147**(1): 27-27.
- Syvanen, M.** (1984). The Evolutionary Implications of Mobile Genetic Elements. *Annual Review of Genetics* **18**: 271-293.
- Tarr, P. I., Bilge, S. S., Vary, J. C., Jelacic, S., Habeeb, R. L., Ward, T. R., Baylor, M. R., and Besser, T. E.** (2000). Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infection and Immunity* **68**(3): 1400-1407.
- Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S., Hayashi, T., and Sasakawa, C.** (2001). *tox*B gene on pO157 of Enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infection and Immunity* **69**(11): 6660-6669.
- Tatsuno, I., Kimura, H., Okutani, A., Kanamaru, K., Abe, H., Nagai, S., Makino, K., Shinagawa, H., Yoshida, M., Sato, K., Nakamoto, J., Tobe, T., and Sasakawa, C.** (2000). Isolation and characterization of mini-Tn5Km2 insertion mutants of enterohemorrhagic *Escherichia coli* O157:H7 deficient in adherence to Caco-2 cells. *Infection and Immunity* **68**(10): 5943-5952.
- te Loo, D. M. W. M., Monnens, L. A. H., van der Velden, T. J. A. M., Vermeer, M. A., Preyers, F., Demacker, P. M. N., van der Heuvel, L. P. W. J., and van Hinsbergh, V. W. M.** (2000). Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood* **95**(11): 3396-3402.
- Tesh, V. L., and O'Brien, A. D.** (1992). Adherence and Colonization Mechanisms of Enteropathogenic and Enterohemorrhagic *Escherichia-Coli*. *Microbial Pathogenesis* **12**(4): 245-254.
- Thompson, J. S., Hodge, D. S., and Borczyk, A. A.** (1990). Rapid Biochemical Test to Identify Verocytotoxin-Positive Strains of *Escherichia-Coli* Serotype O157. *Journal of Clinical Microbiology* **28**(10): 2165-2168.
- Thorpe, C. M., Hurley, B. P., Lincicome, L. L., Jacewicz, M. S., Keusch, G. T., and Acheson, D. W. K.** (1999). Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infection and Immunity* **67**(11): 5985-5993.
- Tkalcic, S., Brown, C. A., Harmon, B. G., Jain, A. V., Mueller, E. P. O., Parks, A., Jacobsen, K. L., Martin, S. A., Zhao, T., and Doyle, M. P.** (2000). Effects of diet on rumen proliferation and fecal shedding of *Escherichia coli* O157:H7 in calves. *Journal of Food Protection* **63**(12): 1630-1636.

Torres, A. G., Giron, J. A., Perna, N. T., Burland, V., Blattner, F. R., Avelino-Flores, F., and Kaper, J. B. (2002). Identification and Characterization of *lpfABCC'DE*, a Fimbrial Operon of Enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **70**(10): 5416-5427.

Torres, A. G., Perna, N. T., Burland, V., Ruknudin, A., Blattner, F. R., and Kaper, J. B. (2002). Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. *Molecular Microbiology* **45**(4): 951-966.

Torres-Medina, A. (1981). Morphologic Characteristics of the Epithelial Surface of Aggregated Lymphoid Follicles (Peyer Patches) in the Small- Intestine of Newborn Gnotobiotic Calves and Pigs. *American Journal of Veterinary Research* **42**(2): 232-236.

Toth, I., Cohen, M. L., Rumschlag, H. S., Riley, L. W., White, E. H., Carr, J. H., Bond, W. W., and Wachsmuth, I. K. (1990). Influence of the 60-Megadalton plasmid on adherence of *Escherichia coli* O157:H7 and genetic derivatives. *Infection and Immunity* **58**(5): 1223-1231.

Tzipori, S., Gunzer, F., Donnenberg, M. S., de Montigny, L., Kaper, J. B., and Donahue-Rolfe, A. (1995). The role of the *eaeA* gene in diarrhoea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. *Infection and Immunity* **63**(9): 3621-3627.

Tzipori, S., Karch, H., Wachsmuth, K. I., RobinsBrowne, R. M., O'Brien, A., Lior, H., Cohen, M. L., Smithers, J., and Levine, M. M. (1987). Role of a 60-megadalton plasmid and shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. *Infection and Immunity* **55**(12): 3117-3125.

Van Donkersgoed, J., Graham, T., and Gannon, V. (1999). The prevalence of verotoxins, *Escherichia coli* O157:H7, and *Salmonella* in the feces and rumen of cattle at processing. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* **40**(5): 332-338.

Vandekar, N., Monnens, L. A. H., Karmali, M. A., and Vanhinsbergh, V. W. M. (1992). Tumor-Necrosis-Factor and Interleukin-1 Induce Expression of the Verocytotoxin Receptor Globotriaosylceramide On Human Endothelial-Cells - Implications For the Pathogenesis of the Hemolytic Uremic Syndrome. *Blood* **80**(11): 2755-2764.

Vazquez-Torres, A., and Fang, F. C. (2000). Cellular routes of invasion by enteropathogens. *Current Opinion in Microbiology* **3**(1): 54-59.

Voitoux, E., Lafarge, V., Collette, C., and Lombard, B. (2002). Applicability of the draft standard method for the detection of *Escherichia coli* O157 in dairy products. *International Journal of Food Microbiology* **77**(3): 213-221.

- Wada, Y., Nakazawa, M., and Kubo, M.** (1994). Natural infection with attaching and effacing *Escherichia coli* (O15) in an adult cow. *Journal of Veterinary Medical Science* **56**(1): 151-152.
- Wadolowski, E. A., Burris, J. A., and O'Brien, A. D.** (1990). Mouse Model For Colonization and Disease Caused By Enterohemorrhagic *Escherichia-Coli* O157:H7. *Infection and Immunity* **58**(8): 2438-2445.
- Wales, A. D., Clifton-Hadley, F. A., Cookson, A. L., Dibb-Fuller, M. P., La Ragione, R. M., Sprigings, K. A., Pearson, G. R., and Woodward, M. J.** (2001). Experimental infection of six-month-old sheep with *Escherichia coli* O157:H7. *Veterinary Record* **148**(20): 630-631.
- Wales, A. D., Clifton-Hadley, F. A., Cookson, A. L., Dibb-Fuller, M. P., Laragione, R. M., Pearson, G. R., and Woodward, M. J.** (2002). Production of attaching-effacing lesions in ligated large intestine loops of 6-month-old sheep by *Escherichia coli* O157:H7. *Journal of Medical Microbiology* **51**(9): 755-763.
- Wales, A. D., Pearson, G. R., Skuse, A. M., Roe, J. M., Hayes, C. M., Cookson, A. L., and Woodward, M. J.** (2001). Attaching and effacing lesions caused by *Escherichia coli* O157:H7 in experimentally inoculated neonatal lambs. *Journal of Medical Microbiology* **50**(9): 752-758.
- Wardlaw, A. C.** (1989). *Practical Statistics for Experimental Biologists*, 1st Edition (Chichester: John Wiley & Sons).
- Weinstein, D. L., Jackson, M. P., Samuel, J. E., Holmes, R. K., and O'Brien, A. D.** (1988). Cloning and Sequencing of a Shiga-Like Toxin Type-Ii Variant From an *Escherichia coli* Strain Responsible For Edema Disease of Swine. *Journal of Bacteriology* **170**(9): 4223-4230.
- Wells, J. G., Shipman, L. D., Greene, K. D., Sowers, E. G., Green, J. H., Cameron, D. N., Downes, F. P., Martin, M. L., Griffin, P. M., Ostroff, S. M., Potter, M. E., Tauxe, R. V., and Wachsmuth, I. K.** (1991). Isolation of *Escherichia coli* Serotype O157:H7 and Other Shiga-Like-Toxin-Producing *Escherichia coli* From Dairy-Cattle. *Journal of Clinical Microbiology* **29**(5): 985-989.
- Whittam, T. S., Wachsmuth, I. K., and Wilson, R. A.** (1988). Genetic-Evidence of Clonal Descent of *Escherichia-Coli* O157-H7 Associated With Hemorrhagic Colitis and Hemolytic Uremic Syndrome. *Journal of Infectious Diseases* **157**(6): 1124-1133.
- Wieler, L. H., Tigges, M., Ebel, F., Schaferkordt, S., Djafari, S., Schlapp, T., Baljer, G., and Chakraborty, T.** (1996). The enterohemolysin phenotype of bovine Shiga-like toxin-producing *Escherichia coli* (SLTEC) is encoded by the EHEC-hemolysin gene. *Veterinary Microbiology* **52**(1-2): 153-164.

- Winsor, D. K., Jr., Ashkenazi, S., Chiovetti, R., and Cleary, T. G.** (1992). Adherence of enterohemorrhagic *Escherichia coli* strains to a human colonic epithelial cell line (T84). *Infection and Immunity* **60**(4): 1613-1617.
- Wray, C., McLaren, I., and Pearson, G.** (1989). Occurrence of attaching and effacing lesions in the small intestine of calves experimentally infected with bovine isolates of verocytotoxic *Escherichia coli*. *Veterinary Record* **125**(14): 365-368.
- Wray, C., McLaren, I. M., Randall, L. P., and Pearson, G. R.** (2000). Natural and experimental infection of normal cattle with *Escherichia coli* O157. *Veterinary Record* **147**(3):65-68.
- Xia, Y. G., D Forsman-Semb, K Uhlin, BE** (2000). Regulatory cross-talk between adhesin operons in *Escherichia coli*: inhibition of type 1 fimbriae expression by the PapB protein. *EMBO Journal* **19**(7): 1450-1457.
- Yamamoto, T., and Exheverria, P.** (1996). Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E-coli* strains pathogenic for humans. *Infection and Immunity* **64**(4): 1441-1445.
- Yarze, J. C., and Chase, M. P.** (2000). *E. coli* O157:H7 - Another waterborne outbreak! *American Journal of Gastroenterology* **95**(4): 1096-1096.
- Yu, J., and Kaper, J. B.** (1992). Cloning and characterisation of the *eae* gene of enterohemorrhagic *Escherichia-coli* O157:H7. *Molecular Microbiology* **6**(3): 411-417.
- Zadik, P. M., Chapman, P. A., and Siddons, C. A.** (1993). Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. *Journal of Medical Microbiology* **39**: 155-158.
- Zhang, W. L., Bielaszewska, M., Liesegang, A., Tschape, H., Schmidt, H., Bitzan, M., and Karch, H.** (2000). Molecular Characteristics and Epidemiological Significance of Shiga Toxin-Producing *Escherichia coli* O26 Strains. *Journal of Clinical Microbiology* **38**(6): 2134-2140.
- Zhao, T., Doyle, M. P., Harmon, B. G., Brown, C. A., Mueller, P. O. E., and Parks, A. H.** (1998). Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *Journal of Clinical Microbiology* **36**(3): 641-647.

Appendix

Publications Arising from this Thesis

Gally, D. L., Naylor, S. W., Low, J. C., Gunn, G. J., Synge, B. A., Pearce, M. C., Donachie, W., and Besser, T. E. (2003). Colonisation site of *E coli* O157 in cattle. *Veterinary Record* **152**(10): 307.

Naylor, S. W., Low, J. C., Besser, T. E., Mahajan, A., Gunn, G. J., Pearce, M. C., McKendrick, I. J., Smith, D. G. E., and Gally, D. L. (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infection and Immunity* **71**: 1505-1512.

Science, Editor's Choice. (2003). Supershedders on the Farm. *Science* **299**: 1487.

RCVS Council elections

2003 - With the year 2003, it is with great pleasure that I announce the results of the 2003 RCVS Council elections. The results are as follows: President: Dr. John Wainman, Vice President: Dr. John Wainman, Treasurer: Dr. John Wainman, Secretary: Dr. John Wainman, Members: Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman.

The results of the elections are as follows: President: Dr. John Wainman, Vice President: Dr. John Wainman, Treasurer: Dr. John Wainman, Secretary: Dr. John Wainman, Members: Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman.

Manuscripts and somatic cell counts

2003 - With the year 2003, it is with great pleasure that I announce the results of the 2003 RCVS Council elections. The results are as follows: President: Dr. John Wainman, Vice President: Dr. John Wainman, Treasurer: Dr. John Wainman, Secretary: Dr. John Wainman, Members: Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman, Dr. John Wainman.

when looking after a baby, so further costs would be incurred.

A further month's work is necessary to pay the RCVS registration fee. If there were, in future, an additional licence to practise fee, as suggested in the Veterinary Surgeons Act review consultation document, this would be likely to generate further expense.

The financial benefits of working additional hours would be reduced by childcare costs.

How will a career break affect the proposed licence to practise?

The current costs of CPD would mean that part-time veterinary surgeons working minimal hours to maintain their experience could not justify the costs of the mandatory 35 hours of annual CPD, plus the RCVS registration fee and licence to practise fee.

Most professions encourage mothers (and other career breakers) to return to work, but these proposals will actively discourage or even preclude veterinary surgeons who have taken a career break from returning to practice on a part-time basis. Given the rising number of female graduates, there will be a shortage of veterinary surgeons in practice if flexible working practices for working mothers are not actively encouraged.

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Haywards Heath, West Sussex RH16 3TG

SIR, - I read the thoughtful letter from Roger Blowey and his colleagues on the RCVS proposals published in a widely circulated document 'Veterinary education and training: a framework for 2010 and beyond'.

VET Trust held a conference in November 2001 on the proposals, at which representatives of the RCVS put forward their views and a number of veterinary surgeons active in different parts of the profession expressed their views.

Any readers who would like a copy of the proceedings of the conference should contact me at the address below.

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RCVS Council elections

SIR, - Within the next few days, it is anticipated that all those entitled to vote in the RCVS Council elections will receive voting papers and candidates' personal statements.

The number of members choosing to vote has been in steady decline in recent years, falling to an all-time low of 13 per cent of the electorate last year. There are many issues currently being discussed by the College which could well have an

effect on veterinary surgeons' working lives for years to come - education proposals and preparations for a new Veterinary Surgeons Act, to name but two.

It is important that Council members should have an appreciation of veterinary life in its widest context; the credibility of the profession is very much at stake.

I urge all members of the College to make a critical appraisal of all the manifestos and, above all, to exercise their right to vote for up to six candidates.

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Colonisation site of *E coli* O157 in cattle

SIR, - *Escherichia coli* O157:H7 causes bloody diarrhoea and potentially fatal systemic sequelae in human beings (Karmali and others 1983, Nataro and Kaper 1998). Importantly, it does not cause clinical disease in cattle, the host species most frequently traced as the source of human infection (Borczyk and others 1987, Besser and others 1999). A key step in protecting public health is to know how and where the bacterium persists in this major animal reservoir, but although *E coli* O157 has been cultured from various sites in the bovine gastrointestinal tract, a defined colonisation site has not been previously identified.

We wish to draw attention to a publication identifying the major site of bovine gastrointestinal site colonisation as the intestinal epithelium adjacent to the rectoanal junction (Naylor and others 2003). *E coli* O157 is present at this site in experimentally and naturally colonised cattle in relatively high numbers, and coats the stool as it is released, leading to a non-random distribution in faeces.

These results were generated by research collaborators from the University of Edinburgh, the Scottish Agricultural College, the Moredun Research Institute, BISS and Washington State University, with funding from DEFRA and supported by the Scottish Executive Environment and Rural Affairs Department and the Wellcome Trust. *E coli* O157 demonstrates a tropism in the bovine gastrointestinal tract which is, as far as we are aware, unique in the bacterial world. This is a major advance in our understanding of the biology of this important human pathogen. Factors responsible for the site specificity of *E coli* O157 need to be elucidated, and the funders and research group believe that there will be considerable potential for the development of rational controls.

In the interim, it is important that those involved in the protection of human health, including veterinarians, farmers and abattoir staff, are aware of these results. At farm level it is clear that a minority of cattle act as carriers of the organism, but veterinarians and stock owners should

recognise the potential for between-animal spread of the organism through routine management practices such as rectal examinations and pregnancy testing. Many abattoirs have already introduced practices to minimise faecal contamination of carcasses but knowledge of the specific site may permit additional risk management procedures. Finally, veterinary research studies should consider their sampling strategies in light of these findings.

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References

- BESSER, T. E., GRIFFIN, P. M. & SLUTSKER, L. (1999) *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. *Annual Review of Medicine* **50**, 355-367
- BORCZYK, A. A., KARMALI, M. A., LIOR, H. & DUNCAN, L. M. (1987) Bovine reservoir for verotoxin producing *Escherichia coli* O157:H7. *Lancet* **i**, 98
- KARMALI, M., PETRIC, M., LIM, C., FLEMING, P. C. & STEELE, B. T. (1983) *Escherichia coli* cytotoxin, haemolytic uremic syndrome, and haemorrhagic colitis. *Lancet* **ii**, 1299-1300
- NATARO, J. P. & KAPER, J. B. (1998) Diarrheagenic *Escherichia coli*. *Clinical Microbiology Review* **11**, 142-201
- NAYLOR, S. W., LOW, J. C., BESSER, T. E., MAHAJAN, A., GUNN, G. J., PEARCE, M. C., MCKENDRICK, I. J., SMITH, D. G. E. & GALLY, D. L. (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonisation of enterohaemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infection and Immunity* **71**, 1505-1512

Mastitis and somatic cell counts

SIR, - We agree with James Booth's contention (VR, January 25, p 119) that there is a need for 'a transparent system providing farmers with regular published evidence of adherence to minimum competent standards' for the measurement of somatic cell counts in milk samples. Moreover, similar systems are required for all of the laboratory procedures used in veterinary medicine; the veterinary specialist advisory group at the Royal College of Pathologists has developed a scheme

Lymphoid Follicle-Dense Mucosa at the Terminal Rectum Is the Principal Site of Colonization of Enterohemorrhagic *Escherichia coli* O157:H7 in the Bovine Host

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***Escherichia coli* O157:H7 causes bloody diarrhea and potentially fatal systemic sequelae in humans. Cattle are most frequently identified as the primary source of infection, and *E. coli* O157:H7 generally colonizes the gastrointestinal tracts of cattle without causing disease. In this study, persistence and tropism were assessed for four different *E. coli* O157:H7 strains. Experimentally infected calves shed the organism for at least 14 days prior to necropsy. For the majority of these animals, as well as for a naturally colonized animal obtained from a commercial beef farm, the highest numbers of *E. coli* O157:H7 were found in the feces, with negative or significantly lower levels detected in lumen contents taken from the gastrointestinal tract. Detailed examination demonstrated that in these individuals the majority of tissue-associated bacteria were adherent to mucosal epithelium within a defined region extending up to 5 cm proximally from the recto-anal junction. The tissue targeted by *E. coli* O157:H7 was characterized by a high density of lymphoid follicles. Microcolonies of the bacterium were readily detected on the epithelium of this region by immunofluorescence microscopy. As a consequence of this specific distribution, *E. coli* O157:H7 was present predominately on the surface of the fecal stool. In contrast, other *E. coli* serotypes were present at consistent levels throughout the large intestine and were equally distributed in the stool. This is a novel tropism that may enhance dissemination both between animals and from animals to humans. The accessibility of this site may facilitate simple intervention strategies.**

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as an important cause of human intestinal disease in developed countries over the past 20 years. In addition to bloody diarrhea, intestinal infection can lead to potentially fatal systemic sequelae resulting from the activity of Shiga toxins. The majority of these infections are caused by *E. coli* O157:H7 (21, 26). This serotype has been frequently isolated from cattle feces, and most human EHEC O157:H7 infections originate, either directly or indirectly, from this source (5, 8). A key step in protecting public health is to know how and where the bacterium persists in this major animal reservoir. Until now, no defined site of colonization by *E. coli* O157:H7 in the bovine gastrointestinal tract (GIT) has been described, beyond an affinity for the large intestine (17).

Enteropathogenic *E. coli* (EPEC) and most EHEC strains are known to carry a locus of enterocyte effacement (LEE) pathogenicity island (24). This locus encodes a type III secretion system that mediates attachment to mucosal epithelial cells. Injection of effector proteins results in intimate attach-

ment and characteristic attaching and effacing (A/E) lesions dependent on intimin and Tir (translocated intimin receptor) (16, 22). *E. coli* O157:H7 intimately attaches to a variety of cell types and tissues in vitro, and a few studies have demonstrated that *E. coli* O157:H7 can form A/E lesions on explants of bovine intestinal tissue (3, 29). Studies by Dean-Nystrom et al. recovered *E. coli* O157:H7 from a range of intestinal sites with associated A/E lesions 3 days after challenge of neonatal calves (13) and 4 days after challenge of fasting weaned calves (14). However, it is not clear whether either of these experimental systems reflects natural carriage. The importance of A/E lesion formation in the colonization of ruminants has been shown by the deletion and complementation of intimin in a sheep persistence model (11). The same intimin-negative strain also had an impaired ability to colonize calves. Despite this evidence, no mucosal adherence of any form has been reported in naturally colonized cattle.

The experimental challenge of healthy weaned calves reproduces natural carriage and does not result in clinical disease; *E. coli* O157:H7 distribution in such animals has been investigated (9, 12, 17). Brown et al. (9) recovered *E. coli* O157:H7 from all sites sampled within the GIT, except the abomasum, with the highest recovery rate in the forestomachs. Cray and Moon (12) also demonstrated a ubiquitous distribution but found the highest numbers in large-intestinal sites. These stud-

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ies suggest a variable distribution with no specific tropism for any site within the bovine GIT. However, Grauke et al. (17) used rumen and duodenal cannulae to sample from live calves, and beyond day 16 neither of these locations harbored *E. coli* O157:H7. This was despite the fact that *E. coli* O157:H7 was present in some of the corresponding fecal samples up to day 34, implying a large-intestinal location. None of these studies demonstrated any evidence of mucosal localization or A/E lesions in persistently shedding animals.

The objective of our study was to examine the patterns of fecal excretion and bacterial GIT localization following experimental challenge with different *E. coli* O157:H7 strains. Our early data revealed that *E. coli* O157:H7 frequently is absent or is isolated in insignificant numbers from the GIT contents at necropsy despite remaining easily demonstrable in the feces (10^3 to 10^5 CFU per g). Significantly, Grauke et al. made a similar observation for sheep colonized for more than 2 weeks (17). We therefore investigated the possibility that *E. coli* O157:H7 persists in either the very terminal portion of the rectum or the anal canal. Due to their intrapelvic location, these sites are not routinely sampled during necropsy and have been overlooked in early sampling procedures. Progressively more detailed analysis of shedding cattle, including a 12-month-old naturally colonized steer, has highlighted a unique tropism for the rectal mucosa adjacent to the recto-anal junction (RAJ). This is a significant and previously overlooked aspect of *E. coli* O157:H7 biology.

MATERIALS AND METHODS

***E. coli* O157:H7 strains.** Strains used for oral inoculation of calves were ZAP 196, ZAP 198, and ZAP 3. ZAP 196 and ZAP 198 were both isolated from the same human patient in Washington state (27). Cattle were established as the source of this outbreak. ZAP 196 possesses Shiga toxin 2 (Stx2), while ZAP 198 has been naturally cured of the Stx2 bacteriophage. They are otherwise indistinguishable as determined by phage typing (both type 32), LEE protein secretion level (25), and pulsed-field gel electrophoresis (both nontypeable). ZAP 3 (Scottish *E. coli* O157:H7 reference laboratory number 659) is a bovine isolate from Red House Dairy (Blackburn, West Lothian, Scotland) that caused a milk-borne human outbreak (2). The strain used for challenge via cohabitation with a shedding calf at Washington State University was WSU 2043. This was originally isolated from an asymptomatic calf and possesses the genes for Stx1, Stx2, and Stx2c. All these strains were selected for spontaneous resistance to nalidixic acid in order to facilitate recovery from GIT contents and tissues. The strain isolated from a naturally colonized 12-month-old steer on a farm in Inverness-shire (Scotland) has been designated ZAP 278. It possesses genes for Stx2 and Stx2c (4). All strains used have been shown by PCR using published primers to possess genes for enterohemolysin (15), intimin- γ (1), EspA (25), and EspB (25).

Calf colonization. Separate experimental calf challenges used either ZAP 3, ZAP 196, or ZAP 198 and were performed at the Moredun Research Institute in either containment level 2 or containment level 3 large-animal housing facilities under Home Office license number 60/02105. Calves were reared conventionally on a farm until at least 2 weeks postweaning and were transported to the Moredun Research Institute, where they were acclimatized for at least 3 days prior to challenge. Prior to challenge fecal samples were taken at least twice from each calf and confirmed negative for *E. coli* O157:H7 by immunomagnetic separation (IMS). At the time of challenge the calves' ages ranged from 8 to 14 weeks. The challenge *E. coli* O157:H7 strain was grown overnight in Luria-Bertani (LB) broth (37°C, with aeration) and diluted in sterile phosphate-buffered saline (PBS) to achieve an inoculum of 10^9 CFU per animal in a total volume of 10 ml. The inoculum was administered to the calves via stomach tube and washed down with 500 ml of sterile PBS. Feces were caught or collected per rectum for culture and bacterial enumeration.

Experimental calf challenges with WSU 2043 were performed at Washington State University by a direct-contact infection model system (6). Briefly, groups of calves were first confirmed to be free of detectable *E. coli* O157:H7 shedding as described above. Then a single calf was removed from the group and experi-

mentally challenged with an inoculum prepared as described for the above calves but administered orally by syringe. The reintroduction of the inoculated calf into the group pen constituted the challenge, and the subject calves were monitored for acquisition of *E. coli* O157:H7 infection.

The 12-month-old steer that was naturally shedding $>10^4$ CFU of *E. coli* O157:H7 per g of feces was identified on a farm in Inverness-shire by field epidemiology work.

Necropsy sampling. The precise procedure varied; more-detailed analysis of the terminal rectum was performed as the tropism of *E. coli* O157:H7 became better defined with each necropsy. The most complete necropsy procedure is described. As close as possible to euthanasia, a sample of naturally passed feces was caught with a fresh glove and was split into surface and core components by removing the surface layer with a sterile scalpel and exposing the core. For certain animals it was not possible to split feces into surface and core components. In these cases whole feces containing an undetermined mixture of surface and core were used for enumeration.

Following euthanasia with intravenous pentobarbital, the abdomen was opened. The terminal 20 to 30 cm of rectum and anus were removed as a single piece after the rectum was double ligated and transected, the anus circumsected, and the pubic bone reflected. This length of gut was opened longitudinally in a proximal-to-distal direction. When present, 10-g samples of lumen contents were taken from between 30 and 20 cm proximal to the RAJ. This junction is grossly visible and is the interface between the colorectal epithelium and anal stratified squamous epithelium. Samples of tissue visibly free of feces were taken from the following portions of rectum relative to the RAJ (in centimeters): 20 to 30, 10 to 20, 5 to 10, 3 to 5, 1 to 3, and -1 to +1. Lumen contents and tissue were also taken from the following locations: rumen (cranioventral sac), ileum (10 cm proximal to the ileo-ceco-colic junction, including both normal absorptive mucosa and Peyer's patches), cecum (apex), and colon (point of inflection of spiral colon). All lumen contents and tissue were processed for microbiological analysis (described below). A sample of each tissue was fixed in 4% paraformaldehyde for cutting of sections to be used for immunofluorescent detection of *E. coli* O157.

Microbiology. Ten-gram quantities of feces or GIT contents were suspended in 90 ml of sterile PBS and serially diluted in 10-fold steps in PBS. Tissue samples were placed in sterile PBS (1 cm² in 5 ml) and vortexed vigorously for 60 s, and the supernatant was diluted in a 10-fold dilution series. These serial dilutions were cultured as 100- μ l aliquots spread in duplicate or triplicate onto sorbitol MacConkey agar (SMAC; Oxoid) plates containing 15 μ g of nalidixic acid (Sigma-Aldrich) ml⁻¹. All inoculated media were incubated overnight at 37°C. Non-sorbitol-fermenting colonies on SMAC plates were counted, and one to three colonies from each sample were tested for O157 lipopolysaccharide antigen by using a latex agglutination test kit (Oxoid). The most probable number of CFU was determined as described under "Statistical methods" below. For enrichment cultures 1 ml of the undiluted suspension or tissue washing supernatant was added to 9 ml of LB broth containing 15 μ g of nalidixic acid ml⁻¹. The enrichment cultures for samples negative on direct plating were spread onto fresh SMAC plates and incubated overnight at 37°C. These procedures are more sensitive than IMS for detecting marked strains (6). Some samples were also spread on tryptone bile X-glucuronide medium (TBX; Oxoid) to enumerate glucuronidase-positive, non-O157:H7 *E. coli* strains (18). Samples for enumeration from the naturally colonized animal were cultured on sorbitol MacConkey agar by using cefixime-tellurite (0.05 and 25 μ g ml⁻¹, respectively; Oxoid) instead of nalidixic acid selection. Similarly, enrichment was performed with LB broth containing cefixime-tellurite in place of nalidixic acid.

Immunofluorescence microscopy. *E. coli* O157 was detected by fluorescence microscopy of paraformaldehyde-fixed sections after incubation with a rabbit anti-O157 polyclonal antibody (Mast-Assure) (1:100 for 30 min at room temperature), followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma-Aldrich) (1:1,000 for 30 min at room temperature). Both antibodies were diluted in PBS containing 0.1% bovine serum albumin. Stained sections were viewed on a Leica DMLB epifluorescent microscope with a 40 \times objective. As a negative control, certain samples were also stained with normal rabbit serum or a polyclonal anti-O26 antibody (Mast-Assure). For confocal microscopy, tissue was counterstained with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin and viewed with a Zeiss 510 confocal microscope with a 63 \times objective lens.

Statistical methods. The most probable number of CFU was determined by fitting generalized linear models with a Poisson error distribution and logarithmic link function while incorporating the logarithm of dilution as an offset variable (23). Bacterial counts at different sites were compared by fitting a generalized linear mixed model (10), with the same error and link functions, while fitting "animal" and "animal site" as random effects. The unbalanced nature of the data did not always allow this model to converge to stable values,

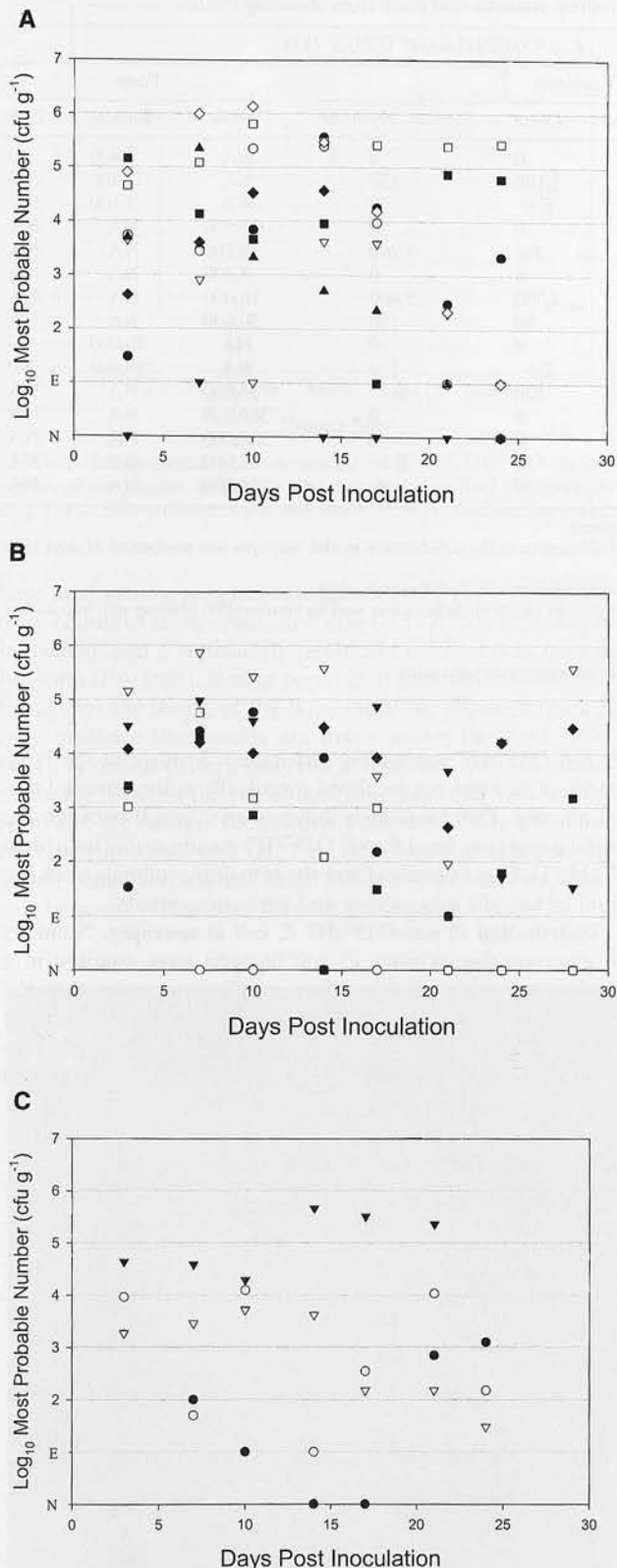


FIG. 1. Scattergraphs illustrating the shedding profiles of three strains, ZAP 196 (A), ZAP 198 (B), and ZAP 3 (C). Each strain was inoculated separately into different calf groups by oral challenge of 10^9 CFU of *E. coli* O157 (Nal^r). Each plot represents the most probable number of *E. coli* O157 per gram of feces. Some of the animals

in which case "animal" was fitted as the sole random effect. These models typically had a moderate to large overdispersion estimate, and these values were always used to estimate the standard errors of the model estimates.

Of the 15 animals studied, the first 4 were not sampled at the full range of sites, and therefore complete data sets were not available for analysis (a random dropout from the sample). A further two animals were identified as exhibiting a colonization pattern qualitatively different from that seen in the others, in that the contents and fecal results suggested that the terminal rectum was not the principal site of colonization (animals 607 and 364). Therefore, only samples from the nine remaining animals were used in the statistical analysis of tissue-associated *E. coli* O157:H7.

RESULTS

Shedding of *E. coli* O157:H7 from orally challenged cattle.

In order to investigate the localization of *E. coli* O157:H7 within the bovine host, it was essential to establish a model that resulted in persistent shedding from healthy weaned animals. By using a number of different strains, persistent shedding for more than 3 weeks was consistently obtained with an oral dose of 10^9 CFU. The shedding patterns for the separate challenges are shown in Fig. 1. Animals for necropsy were chosen from those shedding at 14 to 28 days postinfection.

Distribution of *E. coli* O157:H7 in GIT contents and feces at necropsy. Necropsies were performed on experimentally and naturally colonized animals that were persistently shedding *E. coli* O157. All animals had positive feces, with levels ranging from 10^3 to 10^5 CFU g^{-1} (Table 1). For 13 of the 15 animals, concentrations of *E. coli* O157:H7 were at least 10-fold higher in the feces (antemortem) than in the midrectal contents collected 20 to 30 cm proximal to the RAJ (Table 1). For 11 of the 15 animals, levels of *E. coli* O157:H7 in the feces were at least 100-fold higher than those in either the colon or the midrectal contents. *E. coli* O157:H7 was not recovered from the GIT contents of 6 ruminal, 13 ileal, 7 colonic, and 7 midrectal samples. Statistical analysis showed no evidence of significant differences between samples collected from the colon and the midrectum ($P = 0.99$). However, counts were significantly higher in feces than in both colonic and midrectal contents ($P < 0.001$).

Distribution of *E. coli* O157:H7 on GIT mucosae at necropsy. Tissue-associated *E. coli* O157:H7 bacteria were recovered and enumerated from defined mucosal sites for 11 animals (Table 2). Levels of mucosa-associated *E. coli* O157:H7 on the terminal 5 cm of the rectum were at least 10 times higher than those on any other mucosal surface examined for 9 of the 11 animals. The rumen, ileum, Peyer's patches, colon tissue, and samples 20 to 30 cm from the RAJ, when sampled, typically were negative. Calculation of the mean count for *E. coli* O157:H7 CFU per square centimeter allowed differences between defined tissue sites to be compared statistically. There is clear evidence of an upward trend in counts as samples are taken closer to the RAJ, and the increases are particularly strong between the zones 3 to 5 cm and 1 to 3 cm from the RAJ

represented in these graphs were not shedding at the time of necropsy and therefore were not included in the tables of necropsy results. Samples yielding no colonies on direct culture have been labeled E if enrichment positive and N if enrichment negative to facilitate graphical representation.

TABLE 1. *E. coli* O157:H7 concentrations in gastrointestinal contents and feces from shedding cattle

Calf ^a	Strain	Day ^b	Infection ^c	<i>E. coli</i> O157:H7 concn ^d (CFU g ⁻¹) in:								
				GIT contents					Feces			
				Rumen	Ileum	Cecum	Colon	Rectum, 20–30 cm ^e	Whole	Surface	Core	
351	ZAP 198	21	OD	33	0	0	0	0	0	NA	2,400	0
364	ZAP 198	21	OD	0	0	670	1,100	130	0	NA	1,400	860
360	ZAP 198	20	OD	E+	0	0	E+	0	0	NA	5,100	E+
325	ZAP 278	ND ^f	NC	E+	0	0	0	0	0	33,000	NA	NA
607	WSU 2043	22	CH	E+	550	ND	750	4,200	0	3,200	NA	NA
609	WSU 2043	22	CH	0	0	ND	0	0	0	5,300	NA	NA
611	WSU 2043	22	CH	E+	0	ND	4,700	5,400	0	16,000	NA	NA
612	WSU 2043	22	CH	300	0	ND	50	150	0	30,000	NA	NA
295	ZAP 196	25	OD	0	0	ND	0	0	0	NA	70,000	0
299	ZAP 196	24	OD	E+	0	ND	E+	150	0	NA	280,000	390
307	ZAP 196	14	OD	0	0	ND	520	E+	0	36,000	NA	NA
310	ZAP 198	28	OD	45	0	ND	0	0	0	380,000	NA	NA
323	ZAP 198	28	OD	E+	0	ND	0	0	0	330,000	NA	NA
118	ZAP 3	28	OD	0	0	ND	0	E+	0	2,600	NA	NA
121	ZAP 3	23	OD	0	E+	ND	50	E+	0	230,000	NA	NA

^a Calves are listed in the reverse chronological order in which necropsies were performed.

^b Number of days postinoculation or postexposure at which necropsy was performed. For calves in the cohabitation model, necropsy was performed 22 days after initial exposure to the shedding animal.

^c Source of infection; OD, oral dose of 10⁹ CFU g⁻¹; NC, naturally colonized; CH, cohabitation with shedding individual.

^d Values represent most probable numbers rounded to 2 significant figures. NA, not applicable; either whole feces was used for enumeration or it was split into surface and core components. E+, positive by enrichment culture but not by direct plating.

^e Contents of the rectum within a region 20 to 30 cm proximal to the RAJ.

^f ND, not determined. The naturally colonized animal acquired *E. coli* O157:H7 at an undetermined time point.

($P < 0.001$) and between the zones 1 to 3 cm and -1 to +1 cm from the RAJ ($P = 0.001$) (Fig. 2).

Distribution of *E. coli* O157:H7 within feces. Freshly produced feces from five animals were obtained as the animals defecated and were dissected into surface and core components. *E. coli* O157:H7 bacteria were unevenly distributed in the feces of four animals. For these, the mean surface count was 2.2×10^4 CFU g⁻¹, approximately 1,000-fold higher than the mean core count (2.5×10^1 CFU g⁻¹) (Table 1). This difference is highly statistically significant ($P < 0.001$). In contrast, there was no significant difference between mean counts in the core feces and midrectal contents ($P = 0.82$). In calf 364,

E. coli O157:H7 was widely distributed throughout the large intestine and was not localized specifically at the terminal rectal mucosa. There was little difference (<2-fold) between the surface and core fecal *E. coli* O157:H7 counts in this individual (Table 1). Fecal samples from the remaining animals were too fluid to be split into surface and core components.

Distribution of non-O157:H7 *E. coli* at necropsy. Numbers of glucuronidase-positive *E. coli* bacteria were counted in a random selection of the above samples to ensure that the majority of *E. coli* bacteria within the bovine GIT do not exhibit the same tropism. *E. coli* O157:H7 is excluded from these data because most strains of this serotype, including all

TABLE 2. Tissue-associated *E. coli* O157:H7 levels on mucosae taken from regions of the GIT at necropsy

Calf ^a	Strain	Day ^b	Infection ^c	<i>E. coli</i> O157:H7 concn (CFU cm ⁻²) in the following mucosal sample:									
				Rumen	Ileum	Peyer's patches	Colon	RAJ ^e					
								20 to 30	10 to 20	5 to 10	3 to 5	1 to 3	-1 to +1
351	ZAP 198	21	OD	0	0	0	0	0	0	0	E+	1,100	1,600
364	ZAP 198	21	OD	0	0	0	E+	0	0	0	E+	0	E+
360	ZAP 198	20	OD	0	0	0	0	0	0	0	E+	2,100	9,800
325	ZAP 278	ND ^f	NC	0	0	0	0	0	0	61	83,000	21,000	5,800
607	WSU 2043	22	CH	0	200	ND	230	ND	17	250	33	33	33
609	WSU 2043	22	CH	0	0	ND	ND	ND	0	0	33	50	1,900
611	WSU 2043	22	CH	0	0	ND	ND	ND	950	720	57,000	300,000	29,000
612	WSU 2043	22	CH	0	0	ND	ND	ND	17	1,600	3,300	4,000	16,000
295	ZAP 196	25	OD	ND	ND	ND	ND	ND	30	210	E+	680	100,000
299	ZAP 196	24	OD	ND	ND	ND	ND	ND	30	45	15	620	84,000
307	ZAP 196	14	OD	ND	ND	ND	ND	ND	ND	2,500	ND	ND	190,000

^a Calves are listed in the reverse chronological order in which necropsies were performed.

^b Number of days postinoculation or postexposure at which necropsy was performed.

^c Source of infection; OD, oral dose of 10⁹ CFU g⁻¹; NC, naturally colonized; CH, cohabitation with shedding individual.

^d Values represent most probable numbers rounded to 2 significant figures. E+, positive by enrichment culture but not by direct plating.

^e Numbers indicate proximal distance (in centimeters) from this anatomical boundary.

^f ND, not determined. The naturally colonized animal acquired *E. coli* O157:H7 at an undetermined time point.

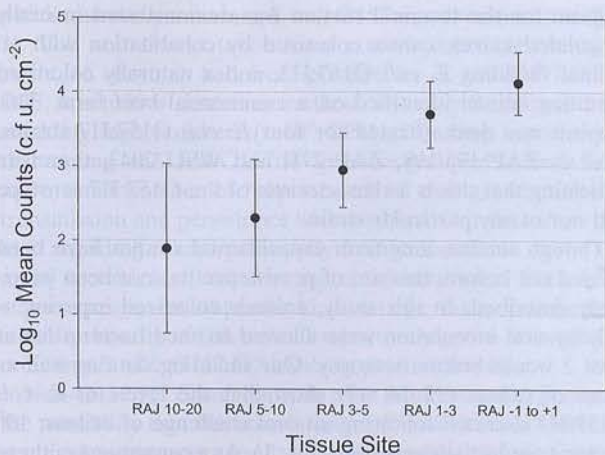


FIG. 2. Mean counts of tissue-associated *E. coli* O157:H7 on five regions of the rectum defined by distance from the RAJ (in centimeters). Error bars indicate upper and lower 95% confidence intervals.

those in this study, are glucuronidase negative (30). The lowest mean counts of tissue-associated non-O157 *E. coli* occurred in the rumen, and a statistically significant increase was seen in the ileum ($P = 0.01$). Similar population sizes were maintained throughout the length of the large intestine, although there is some evidence that counts are lower nearer the RAJ ($P < 0.001$). A similar pattern was seen in contents, with the counts increasing between the rumen and the ileum and between the ileum and the cecum. There was no statistical evidence of any difference in mean non-O157 *E. coli* numbers between the cecum, colon, rectum, and feces (all $P > 0.05$) and no difference between surface and core fecal samples ($P = 0.47$).

Detection of adherent *E. coli* O157 by microscopy. Adherent *E. coli* O157 bacteria were readily detected on the epithelial surface of the terminal rectal mucosa by fluorescence microscopy using anti-O157 immunostaining. Microcolonies of *E. coli* O157 were detected on sections with corresponding tissue-associated bacterial counts of 2×10^3 CFU cm⁻² or greater. This included demonstration of *E. coli* O157 microcolonies on the rectal mucosa from the naturally colonized animal (calf 325) (Fig. 3). No fluorescent bacteria were observed when either normal rabbit serum or an anti-O26 antibody was used in place of the anti-O157 antibody.

Characterization of the mucosa of the terminal rectum. In gross appearance, the smooth raised mucosa adjacent to the RAJ contrasted subtly with the folded surface of the normal absorptive rectal mucosa. This characteristic was reminiscent of the Peyer's patches of the bovine ileum. Histologically the RAJ (Fig. 4A) is the clear junction between the stratified squamous epithelium of the anal canal and the columnar epithelial mucosa of the rectum. Large aggregates of submucosal lymphoid follicles were visible in hematoxylin-and-eosin-stained sections of the rectal mucosa adjacent to the RAJ (Fig. 4). These follicles extended for 2 to 5 cm proximal to the RAJ and, at their apexes, penetrated the muscularis mucosa to contact areas of epithelium.

DISCUSSION

This research demonstrates that *E. coli* O157:H7 exhibits a novel tropism for the terminal rectum in the bovine host. In almost all persistently colonized animals, the majority of tissue-associated bacteria were identified in a region within 5 cm, or in many cases 3 cm, proximal to the RAJ (Fig. 4A). The

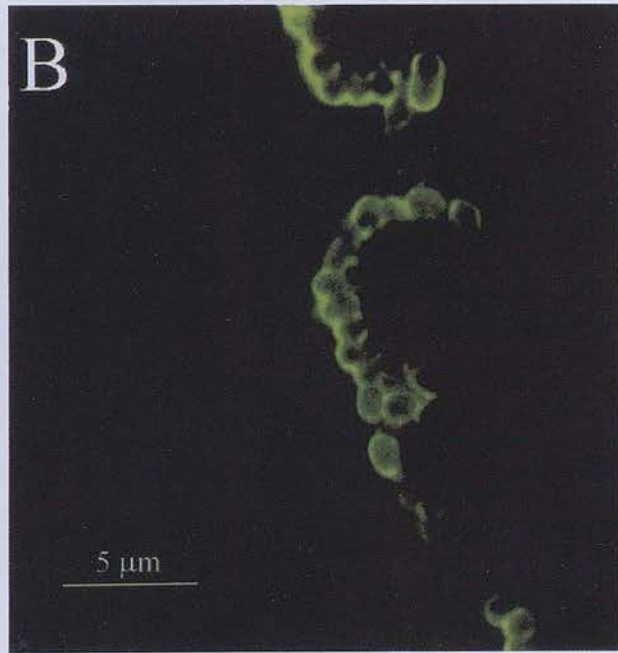
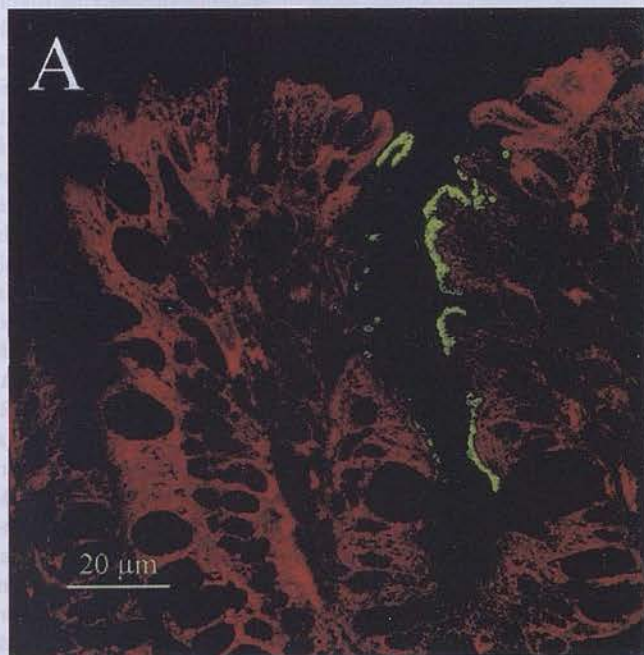


FIG. 3. Confocal micrographs of a microcolony of *E. coli* O157 on the epithelium of the terminal rectum from calf 325. (A) Pseudocrypt lined with adherent *E. coli* O157:H7 (green), with tissue counterstained with TRITC-phalloidin (red) (63 \times objective). (B) A different Z-plane from a region of the sample featured in panel A that has been digitally magnified by a factor of 6 by using the Zeiss 510 confocal image software.

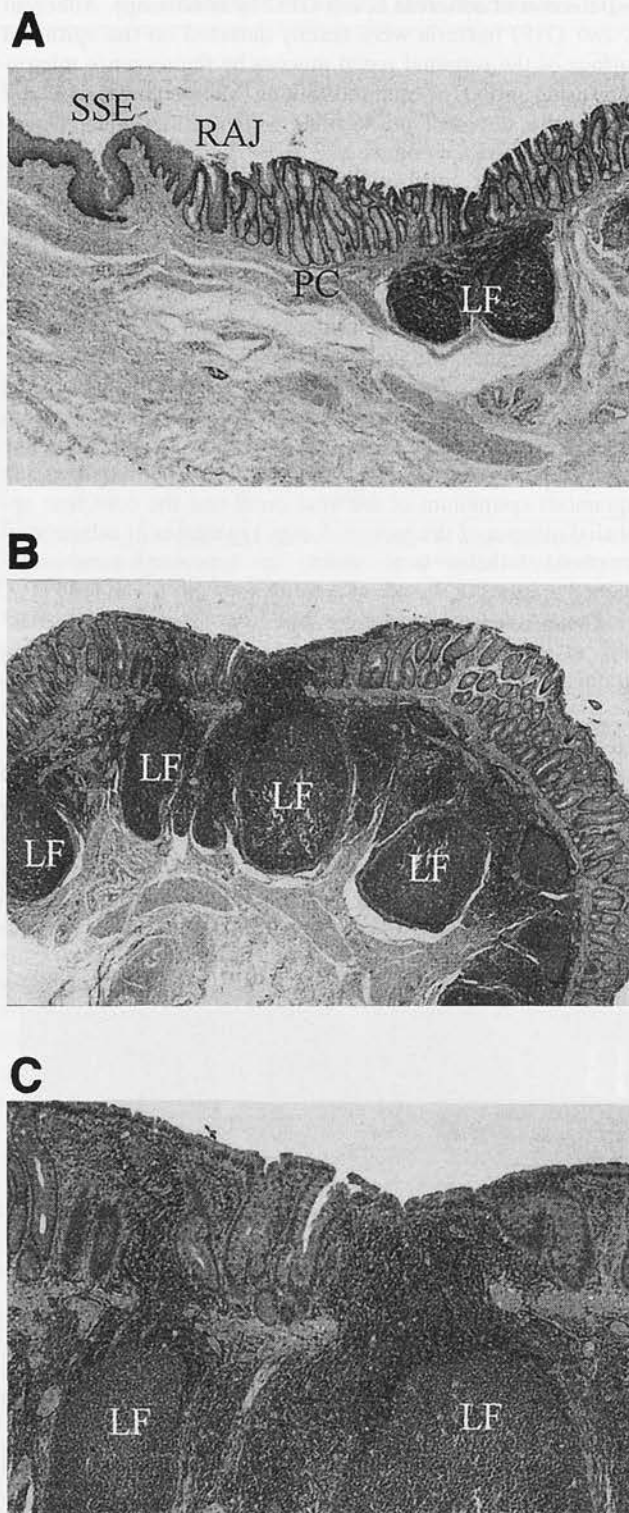


FIG. 4. Hematoxylin-and-eosin-stained sections. (A) The RAJ, defined as the junction between stratified squamous epithelium (SSE) and the columnar epithelium folded into pseudocrypts (PC) which lines the large intestine. Two lymphoid follicles (LF) are also visible ($2.5\times$ objective). (B and C) Terminal rectal mucosa from within 1 cm of the RAJ. Panel B demonstrates the typical density of lymphoid follicles found in this region ($2.5\times$ objective). Panel C highlights a lymphoid follicle in contact with an area of epithelium notable by the absence of goblet cells ($10\times$ objective).

tropism for the terminal rectum was demonstrated in orally inoculated calves, calves colonized by cohabitation with an animal shedding *E. coli* O157:H7, and a naturally colonized shedding animal identified on a commercial beef farm. This tropism was demonstrated for four *E. coli* O157:H7 strains, ZAP 3, ZAP 196/198, ZAP 278, and WSU 2043, therefore indicating that this is a characteristic of the O157:H7 serotype and not of any particular strain.

Though similar, long-term experimental studies have been carried out before, this site of persistence has not been previously described. In this study, animals colonized experimentally by oral inoculation were allowed to shed bacteria for at least 2 weeks before necropsy. Our shedding data as well as those of others (12–14, 17) show that the levels of *E. coli* O157:H7 excreted following an oral challenge of at least 10^9 bacteria are initially very high (Fig. 1). As a consequence, there is an increased probability of observing a widespread distribution if examination of bacterial localization is carried out early after challenge. Waiting for at least 2 weeks allowed the bacteria to establish a long-term persistence that is similar in duration to the natural carriage described by Besser et al. (7). Interestingly, Grauke et al. (17) stated that for 10 sheep from whose feces the bacteria were easily cultured, after more than 2 weeks following inoculation, the organism could not be found in any GIT tissue or other digesta. In another experiment within the same study, the only tissue sample from a sheep persistently colonized beyond day 15 from which *E. coli* O157:H7 was cultured was taken from the rectum, although it is not stated whether it was from the terminal rectum. It is important that in no previous studies has there been sampling specifically from the terminal rectum, and presumably the possibility of carriage at this site has been overlooked.

For 13 of the 15 animals persistently shedding *E. coli* O157:H7, there was a significant increase in bacterial numbers present in the feces over those in samples of contents taken from various sites in the GIT (Table 1). There were also high levels of tissue-associated bacteria immediately proximal to the RAJ (Table 2). As a consequence there was an uneven distribution of *E. coli* O157:H7 in the fecal stool, since the bacteria coat only the surface as it is released from the rectum. *E. coli* O157:H7 microcolonies could be readily detected on sections taken from the terminal rectum, with bacteria adherent to the apical surfaces of epithelial cells (Fig. 3). Of the 15 animals examined in the study, only 3 (animals 364, 607, and 611) (Table 1) demonstrated evidence that *E. coli* O157:H7 bacteria from sites other than the terminal rectum were contributing to more than 10% of the fecal load of the organism. These animals may have been colonized by *E. coli* O157:H7 at a mucosal site(s) other than the terminal rectum, or the organism could simply be present within the GIT digesta. Reingestion from a contaminated environment may contribute to persistence in either case. Colonization at the terminal rectum has the greatest impact on the numbers of bacteria shed, but as the dynamics of persistence at the terminal rectum have not been explored, there is a possibility that this persistence is dependent on recolonization. This may account for the fluctuations in numbers of shed *E. coli* O157:H7 bacteria observed in our shedding data and demonstrated in other studies (12).

E. coli O157:H7 was present at the terminal rectum in the naturally colonized animal (animal 325) (Tables 1 and 2) in

which mucosally adherent *E. coli* O157 was demonstrated (Fig. 3). This animal was taken from a straw court containing 35 cattle, 16 of which had feces positive for *E. coli* O157:H7 by IMS, a sensitive detection method, but only 3 of these were shedding high numbers ($>10^4$ CFU g⁻¹). One hypothesis is that among an *E. coli* O157:H7-positive group of animals there are a small number of "supershedders" that greatly enhance transmission and persistence within a herd. Our data show that high shedding results principally from colonization at the terminal rectum, and therefore rectal colonization is likely to be a prerequisite for supershedders on the farm. Intervention aimed primarily at this animal subset may prove successful in reducing *E. coli* O157:H7 levels in positive herds.

Non-O157:H7 *E. coli* bacteria were enumerated to eliminate the possibility that the association of *E. coli* O157:H7 with the terminal rectum was a result of a rapid multiplication of enteric bacteria in response to environmental factors. The non-O157:H7 *E. coli* organisms were present throughout the gut and reached their highest levels in the large intestine. Importantly, no increases were observed at the RAJ and fecal surface. Therefore, the majority of *E. coli* bacteria in the bovine gut do not share the *E. coli* O157:H7 tropism for the terminal rectum.

Bacterial tropism for this site has not been previously described, and the factors that drive it are unknown. It is likely to be based on the interaction between a bacterial adhesin and an epithelial cell receptor, access or expression of which occurs predominantly in the terminal rectum. Our analysis of this region has shown it to contain a high concentration of lymphoid follicles. For both sheep (31) and humans (28) the RAJ has also been described as an area rich in lymphoid follicles. The apparent tropism for this site exhibited by *E. coli* O157:H7 may be related to this feature, since other members of the *Enterobacteriaceae*, e.g., *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, *Shigella* spp., and *Yersinia* spp. (20, 32), have well-described tropisms for Peyer's patches, another region of gut-associated lymphoid tissue. There is some evidence that intimin- γ , which is expressed by EHEC O157:H7, mediates a specificity for follicle-associated epithelium of human Peyer's patches (29) and shows more-restricted tissue affinity than do other intimin types (19). A recent study (11) has shown the importance of intimin to the persistence of *E. coli* O157:H7 in ruminant colonization, and our ongoing studies do support a role for type III secretion and intimate attachment at the terminal rectum of the bovine host. With the knowledge of the site of persistence in the bovine host, it will be feasible to examine the host-pathogen interactions directly. An understanding of the cellular interactions that allow *E. coli* O157:H7 to persist on the rectal mucosae should lead to the development of rational control strategies.

Worldwide, among shiga-toxigenic *E. coli* (STEC) serotypes, *E. coli* O157:H7 is the predominant human pathogen. Why this serotype is so relatively successful at causing disease has been an enigma, since the virulence factors identified to date are shared by many other STEC serotypes. Our preliminary studies with *E. coli* serotypes O26, O111, and O5, also associated with human disease, indicate that they do not share a tropism for the bovine terminal rectum. However, abattoir surveys are required to confirm the contribution of this tropism to the maintenance of *E. coli* O157:H7 and other STEC serotypes in

cattle populations. These results will have substantial implications for research aimed at understanding the biology of this important food-borne organism. It is possible to envisage treatments aimed at eradicating the bacteria from the site, and such control strategies, whether applied at a farm or an abattoir level, could significantly benefit human health.

ACKNOWLEDGMENTS

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REFERENCES

1. AduBobie, J., G. Frankel, C. Bain, A. G. Goncalves, L. R. Trabulsi, G. Douce, S. Knutton, and G. Dougan. 1998. Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. *J. Clin. Microbiol.* **36**:662-668.
2. Allison, L., A. Stirrat, and F. M. Thompson-Carter. 1998. Genetic heterogeneity of *Escherichia coli* O157:H7 in Scotland and its utility in strain subtyping. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**:844-848.
3. Baehler, A. A., and R. A. Moxley. 2000. *Escherichia coli* O157:H7 induces attaching-effacing lesions in large intestinal mucosal explants from adult cattle. *FEMS Microbiol. Lett.* **185**:239-242.
4. Bastian, S. N., I. Carle, and F. Grimont. 1998. Comparison of 14 PCR systems for the detection and subtyping of *stx* genes in Shiga-toxin-producing *Escherichia coli*. *Res. Microbiol.* **149**:457-472.
5. Besser, R. E., P. M. Griffin, and L. Slutsker. 1999. *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. *Annu. Rev. Med.* **50**:355-367.
6. Besser, T. E., B. L. Richards, D. H. Rice, and D. D. Hancock. 2001. *Escherichia coli* O157:H7 infection of calves: infectious dose and direct contact transmission. *Epidemiol. Infect.* **127**:555-560.
7. Besser, T. E., D. D. Hancock, L. C. Pritchett, E. M. McRae, D. H. Rice, and P. I. Tarr. 1997. Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *J. Infect. Dis.* **175**:726-729.
8. Borczyk, A. A., M. A. Karmali, H. Lior, and L. M. C. Duncan. 1987. Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* **i**:98.
9. Brown, C. A., B. G. Harmon, T. Zhao, and M. P. Doyle. 1997. Experimental *Escherichia coli* O157:H7 carriage in calves. *Appl. Environ. Microbiol.* **63**:27-32.
10. Brown, H., and R. Prescott. 1999. Applied mixed models in medicine. Wiley and Sons, Chichester, England.
11. Cornick, N. A., S. L. Booher, and H. W. Moon. 2002. Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. *Infect. Immun.* **70**:2704-2707.
12. Cray W. C., Jr., and H. W. Moon. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **61**:1586-1590.
13. Dean-Nystrom, E. A., B. T. Bosworth, W. C. Cray, and H. W. Moon. 1997. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infect. Immun.* **65**:1842-1848.
14. Dean-Nystrom, E. A., B. T. Bosworth, and H. W. Moon. 1999. Pathogenesis of *Escherichia coli* O157:H7 in weaned calves. *Adv. Exp. Med. Biol.* **473**:173-177.
15. Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic. 1999. Detection of Shiga toxin (*stx1* and *stx2*), intimin (*eaeA*), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. *Appl. Environ. Microbiol.* **65**:868-872.
16. Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutton. 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol. Microbiol.* **30**:911-921.
17. Graue, L. J., I. T. Kudva, J. W. Yoon, C. W. Hunt, C. J. Williams, and C. J. Hovde. 2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. *Appl. Environ. Microbiol.* **68**:2269-2277.
18. Hansen, W., and E. Yourassowsky. 1984. Detection of beta-glucuronidase in lactose-fermenting members of the family *Enterobacteriaceae* and its presence in bacterial urine cultures. *J. Clin. Microbiol.* **20**:1177-1179.
19. Hartland, E. L., V. Huter, L. M. Higgins, N. S. Goncalves, G. Dougan, A. D. Phillips, T. T. MacDonald, and G. Frankel. 2000. Expression of intimin γ from enterohemorrhagic *Escherichia coli* in *Citrobacter rodentium*. *Infect. Immun.* **68**:4637-4646.

20. Jensen, V. B., J. T. Harty, and B. D. Jones. 1998. Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. *Infect. Immun.* **66**:3758-3766.
21. Karmali, M., M. Petric, C. Lim, P. Fleming, and B. Steele. 1983. *Escherichia coli* cytotoxin, hemolytic uremic syndrome and hemorrhagic colitis. *Lancet* **ii**:1299-1300.
22. Kenny, B., R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay. 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**:511-520.
23. McCullagh, P., and J. A. Nelder. 1989. Generalised linear models, 2nd ed. Chapman and Hall, London, England.
24. McDaniel, T. K., and J. B. Kaper. 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol. Microbiol.* **23**:399-407.
25. McNally, A., A. J. Roe, S. Simpson, F. M. Thomson-Carter, D. E. E. Hoey, C. Currie, T. Chakraborty, D. G. E. Smith, and D. L. Gally. 2001. Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157:H7. *Infect. Immun.* **69**:5107-5114.
26. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**:142-203.
27. Ostroff, S. M., P. M. Griffin, R. V. Tauxe, L. D. Shipman, K. D. Greene, J. G. Wells, J. H. Lewis, P. A. Blake, and J. M. Kobayashi. 1990. A statewide outbreak of *Escherichia coli* O157:H7 infections in Washington State. *Am. J. Epidemiol.* **132**:239-247.
28. Parks, A. G., and B. C. Morson. 1962. Fistula-in-ano. *Proc. R. Soc. Med.* **55**:751-754.
29. Phillips, A. D., S. Navabpour, S. Hicks, G. Dougan, T. Wallis, and G. Frankel. 2000. Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* **47**:377-381.
30. Ratnam, S., S. B. March, R. Almed, G. S. Bezanon, and S. Kasatiya. 1988. Characterisation of *Escherichia coli* serotype O157:H7. *J. Clin. Microbiol.* **26**:2006-2012.
31. Sedgmen, B. J., S. A. Lofthouse, J. P. Y. Scheerlinck, and E. N. T. Meeusen. 2002. Cellular and molecular characterisation of the ovine rectal mucosal environment. *Vet. Immunol. Immunopathol.* **86**:215-220.
32. Vazquez-Torres, A., and F. C. Fang. 2000. Cellular routes of invasion by enteropathogens. *Curr. Opin. Microbiol.* **3**:54-59.

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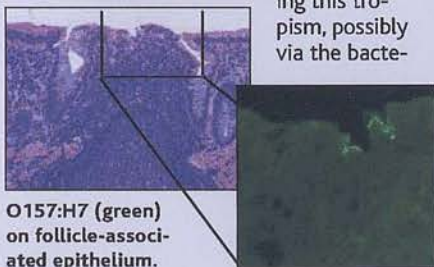
vitro model, binding of VacA to Ptpz caused epithelial cells to detach from the basement membrane, suggesting that ulcers may form because the interaction of these proteins exposes the denuded gastric mucosa to the acidic contents of the stomach. Further study of the cellular pathways affected by VacA will likely facilitate the development of vaccines and diagnostic tests for identifying individuals with a high risk of gastric disease. — PAK

Nature Genet. 10.1038/ng1112 (2003).

MICROBIOLOGY

Supershedders on the Farm

Escherichia coli O157:H7 produce Shiga toxins, which cause bloody diarrhea and potentially fatal systemic infections in humans. Cattle are thought to be the main source of these dangerous organisms, but they themselves show few symptoms of infection. Naylor *et al.* surveyed four strains of O157:H7 in experimentally infected calves. Most bacteria were found in feces, and after necropsy it was observed that the recto-anal tissues contained adherent colonies of O157:H7. These tissues also have a high density of lymphoid follicles, which may be governing this tropism, possibly via the bacter-



O157:H7 (green) on follicle-associated epithelium.

rial adhesion protein intimin. By contrast, other *E. coli* serotypes were distributed evenly throughout the large intestine. This is a novel tissue tropism for a pathogen, which appears to be an adaptation to maximizing transmission among natural hosts and one that could also allow ready diagnosis and treatment of the carrier status of a herd. — CA

Inf. Immun. 71, 1505 (2003).

CLIMATE SCIENCE

Acid Rain = Alkaline Lake

Acid rain has dramatically altered the chemistry of several watersheds and lakes in New England, the western United States, and parts of Europe, particularly in areas where the buffering capacity of

soils is low. Many such areas have been studied over the past several decades. But what has the impact been in drainages and lake basins (generally rich in limestone) that are covered in soils with a high pH or buffering capacity? It has been thought that in these areas the impact would be much less or minimal.

Lajewski *et al.* examined this question by studying sediments in the Finger Lakes region of New York, an area that has received copious amounts of acid rain during the 20th century and that has soils with a high buffering capacity. They found that in several of the lakes, authigenic calcite, which had been absent in the sediments for more than 4000 years, began precipitating during the 1970s. Apparently, acid rain in the region, perhaps augmented by soil disturbances, has increased the weathering of carbonate rocks and thus changed the saturation state of carbonate in the lakes. Paradoxically, acid rain in such regions may make waters more basic. — BH

Geol. Soc. Am. Bull. 115, 373 (2003).

CHEMISTRY

Making a Glowing Polymer

Aluminum tris(8-hydroxyquinoline) or Alq₃ is one of the more stable fluorescent solid-state materials; hence, it is commonly used as the emission and electron transport layers in organic light-emitting diodes (OLEDs). However, the need to deposit Alq₃ in vacuum is incompatible with the current trend toward fabricating OLEDs via a solution process. One option has been to trap Alq₃ within a polymer matrix, and another route has been to couple it to the polymer backbone after polymerization, but these approaches have shown only limited success to date.

Instead, Meyers and Weck have designed a functionalized monomer containing Alq₃. Norbornene was chosen as the polymer backbone because it can be polymerized by ring-opening metathesis, a versatile method that has a high tolerance for many functional groups. The pure polymer exhibited only limited solubility, but this could be remedied by incorporating an aliphatic-norbornene comonomer. The photoluminescence emission spectra of the copolymer in solution was similar to that of the parent Alq₃, and preliminary studies of spin-cast films indicate that this is also the case for the solid-state copolymer. — MSL

Macromolecules 10.1021/ma0259012 (2003).