Escherichia coli O157:H7 colonisation in cattle: mucosal pathology and immune responses

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Abstract

Escherichia coli O157:H7 causes serious human disease including haemorrhagic colitis and haemolytic uraemic syndrome. Ruminants and in particular cattle are a reservoir of infection, though colonised animals are clinically asymptomatic. Recent research has established that the terminal rectum is the predominant colonisation site for enterohaemorrhagic *E. coli* O157:H7 in cattle. Thus the main goals of the PhD project were to understand the carriage and persistence of *E. coli* O157:H7 in the bovine host, through examination of faecal shedding patterns and gastrointestinal tract tissues in experimentally infected calves. A marked bacterial tropism for the terminal rectum was confirmed while other minor carriage sites were identified. *E. coli* O157:H7 induced histopathological alterations of the rectal mucosa and cannot be considered as a commensal. The pathological changes included a local infiltration of neutrophils and production of rectal mucosal IgA responses against *E. coli* O157:H7 antigens. The work presents evidence of strong local IgA immune responses directed against membrane protein components, Type III-secreted proteins, O157 lipopolysaccharide and H7 flagellin. This study has sought to elucidate the specifc mucosal immune responses against *E. coli* O157:H7 outer membrane porin C, and in particular, against the sections of the protein situated on the external surface of the bacterial membrane. While there were consistent immune responses against the whole membrane protein, purified peptides of the external areas of the porin failed to elicit detectable mucosal immune responses. The identification of pathological changes and local host responses to *E. coli* O157:H7 colonisation of the

terminal rectum confirms that pathological changes previously reported with *E. coli* O157 in the intestine, are also found in the rectum. The results offer the first step in the development of a means to control carriage of this organism by cattle. The results will be important for the development of diagnostic tests and potentially for vaccine control methods.

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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.

Abbreviations

AE	Attaching and effacing lesions
AggR	Aggregative Regulon
APEC	Avian Pathogenic *Escherichia coli*
BFP	Bundle-forming pilus
CFU	Colony-forming unit
1D	One-dimensional
2D	Two-dimensional
DAEC	Diffusely adherent *Escherichia coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
ECNM	*Escherichia coli* Neonatal Meningitis
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal pathogenic *Escherichia coli*
H&E	Haematoxylin and eosin
HUS	Haemolytic uraemic syndrome
Ig	Immunoglobulin
IMS	Immuno Magnetic Separation
kb	Kilo base pair
kD	Kilodalton
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharide
LT	Heat-label toxin
MALDI ToF-ToF	Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight
MAP	Mitochondrial associated protein
MHC	Major Histocompatibility Complex
OmpC	Outer Membrane Porin C
Pet	Plasmid-encoded toxin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis,
ShET1	*Shigella* Enterotoxin 1
PAIs	Pathogenecity islands
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis,
QseA	*Eschrichia coli* regulator A
RAJ	Recto-anal junction
SEM	Scanning Electron Microscopy
SS	Secretion System
ST	Heat-stable toxin
STEC	Shiga toxin producing *Escherichia. coli*
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
TBF	Tris-buffered Ficoll
TEM	Transmission Electron Microscopy
Tir	Translocated intimin receptor

TR	Terminal Rectum
TTSS	Type Three Secretion System
UPEC	Uropathogenic *Escherichia coli*
VFs	Virulence factors

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Chapter 1

Introduction

1.1. *Escherichia coli*

Escherichia coli are phylogenetically one of the 130 species of the family *Enterobacteriaceae*, phylum *Bacteroides*. Structurally they are Gram negative, rod-shaped bacteria, propelled by flagella and capable of expressing fimbriae. Biochemically they are facultative anaerobes, capable of growing fermentatively on carbohydrates. *E. coli* is classified by lipopolysaccharide (O) and flagellar (H) antigens that define serogroups (O antigen only) or serotypes (O and H antigens) (Garrity et al., 2001). *E. coli* are a genetically diverse species that includes non-pathogenic and pathogenic variants. *E. coli* is a consistent member of the normal flora of a mammalian gastrointestinal tract, and in particular of the mucus layer of the colon. In the immunosuppressed host or when gastrointestinal barriers are breached commensal strains of *E. coli* can cause infection. Moreover, a number of *E. coli* strains have acquired several virulence factors capable of producing a variety of pathological changes in animals and humans.

Both diversity and pathogenicity are the result of two evolutionary processes: mutation and lateral DNA transfer (Ochman and Davalos, 2006). Mutation is the sporadic introduction of single-nucleotide substitutions or deletions of various sizes in the genome, which are transmitted vertically to successive generations, and result in a clonal population (Denamur and Matic, 2006). Horizontal transfer involves the exchange of DNA between different *E. coli* strains or between another species and *E. coli*. Three mechanisms are responsible for the movement of DNA between bacteria: (i) conjugation mediated by a pilus and usually

of extrachromosomal DNA such as a plasmids (ii) transduction by viruses commonly resulting in integration of pathogenic determinants of toxins, persistence or immune modulation factors, and (iii) transformation or uptake of external DNA from the environment (Thomas and Nielsen, 2005). The transfer DNA can become incorporated in the recipient. As a consequence, lateral transmissions produce genetic similarities between distinct branches of the phylogenetic tree, thus making possible similar pathological mechanisms in phylogenetically unrelated bacterial clones (Wirth et al., 2006).

Different clinical syndromes are usually recognised: pneumonias/sepsis in poultry, meningitis/sepsis and urinary tract infection in humans and diarrhoea in a variety of mammal species (Kaper et al., 2004). These syndromes can be associated with different *E. coli* pathotypes, combining different *E. coli* lineages that use a common set of virulence factors (Marrs et al., 2005). The first three extraintestinal syndromes mentioned above are caused by one pathotype each, while different pathotypes can cause diarrhoea in a variety of mammal species (Stenutz et al., 2006). Efforts to elucidate the factors involved in the pathogenesis of these infections of considerable medical importance are discussed in subsequent sections.

1.1.1. Diarrhoeagenic Escherichia coli

Although some pathotypes cause disease at extra-intestinal sites, e.g. Uropathogenic *E. coli* (UPEC) (Svanborg et al., 2006), Avian Pathogenic *E. coli* (APEC) (Ron, 2006) and *E. coli* Neonatal Meningitis (NUMEC) (Bonacorsi and Bingen, 2005), most Extraintestinal Pathogenic *E. coli*

strains (ExPEC) are opportunistic, and the site of invasion is not the site of initial colonisation. In contrast, the majority of *E. coli* enteric pathotypes enter the host with food and are capable of colonising the intestines causing diarrhoea (Nataro and Kaper, 1998).

Intestinal pathogenic strains of *E. coli*, are divided into six different pathotypes based on their shared disease mechanisms: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC) and enterohaemorrhagic (EHEC). Common attributes of mucosal pathogenic bacteria are the ability to colonise the host epithelial surface, multiply and cause damage (Robins-Browne and Hartland, 2002). *E. coli* diarrhoeagenic strains have a diverse range of adhesive structures to facilitate colonisation ranging from fimbrial structures (all types) to a highly developed attaching system, (EPEC and EHEC) requiring a type III secretion system (TTSS). Following successful colonisation the strategies of pathogenic *E. coli* to multiply and cause diarrhoea extend from enterotoxin production (EAEC and ETEC), to invasion (EIEC) or effacement of the enterocyte microvilli (EPEC and EHEC) (Nataro and Kaper, 1998). The different categories of diarrhoeagenic *E. coli* and their main characteristics will be discussed in detail in this section.

Enteropathogenic *E. coli* was the first pathotype to be described (Bray, 1945), and is an important cause of infantile diarrhoea in developing countries (Simpson et al., 2006). EPEC strains are also a common cause of diarrhoea in rabbits, pigs and ruminants (Wales et al., 2005a). To colonise the gastrointestinal tract EPEC binds tightly to the host

intestinal mucosa by way of a type three secretion system (TTSS, the mechanism is described in more detail in section 1.2.1) causing characteristic attaching and effacing (AE) lesion. In addition a type IV pilus called the bundle-forming pilus (BFP) mediates interbacterial adherence (Nougayrede et al., 2003). AE lesions are characterised by destruction of the enterocyte apical microvilli, alterations of the cytoskeleton and electrolyte transport of the host cell, which leads to increased permeability and loosening of the tight junctions (Stein et al., 1996; Yuhan et al., 1997). Recent research has determined that EPEC inhibits the Na^+-Glucose cotransporter (SGLT-1) in a TTSS dependent manner in biopsy explants (Dean et al., 2006). SGLT-1 is considered the major water pump in the small intestine and accounts for up to 50% of the daily uptake of fluid (Loo et al., 2002). The inactivation by EPEC of this water absorption system could result in a potent diarrhoeagenic mechanism, in particular considering that an increase of just 10-15% of stool water contents is enough to produce diarrhoea in humans (Fischer et al., 2001). The identification of this mechanism could explain the resultant onset of watery diarrhoea after EPEC intestinal colonisation, although further work *in vivo* is needed to fully validate this finding.

Enterotoxigenic *E. coli* infection is the most common type of colibacillosis of piglets and neonatal calves, and it is the main cause of diarrhoea among travellers and children in the developing world (Nagy and Fekete, 2005; Qadri et al., 2005). For the three species, colonisation, without major intestinal morphological change, is mediated by fimbriae, and secreted enterotoxins are responsible for increased in intestinal secretion. In pigs, the most common pathogenic strains are O138, O139

and O141. Their attachment to the porcine gastrointestinal tract is mediated by F16ab fimbriae and their main virulence factor is Shiga toxin 2e (Stx 2e). The action of Stx2e results in damage to endothelial cells lining the small arteries and arterioles of the gut and brain causing oedema in the submucosa of the gut and infarction and malacia in the brain stem (Moxley, 2000). In calves and humans the toxins produced by ETEC are classified into two groups: heat-labile (LT) and heat-stable (ST) enterotoxins (Al-Majali et al., 2000a; Wolf, 1997). ETEC strains might express LT or ST or both. While STa is mostly isolated from calves (Al-Majali et al., 2000b), diarrhoea caused by ETEC in humans has much in common with cholera illness. LT toxin, found predominantly in human ETEC isolates, is 80% homologous at the amino acid level to cholera toxin (Spangler, 1992). The A subunit is responsible for the enzymatic activity of the toxin, by permanently activating the adenylate cyclase leading to increased secretion of chloride from mucosal epithelial cells (Sears and Kaper, 1996). A number of variants of the ST toxin can cause diarrhoea by activating guanylate cyclase and resulting in chloride and bicarbonate secretion (Vaandrager et al., 2000).

Enteroinvasive *E. coli* share identical pathogenesis to *Shigella* species and together they form the distinctive Shigella/EIEC pathotype (Ina et al., 2003). *Shigella* strains, historically classified in a different genus because of their medical importance, are currently considered *E. coli* clones (Lan and Reeves, 2002; Pupo et al. 2000). Unlike other EIEC strains, *Shigella* species carry Shiga toxin type 1 (Stx 1) (Hale, 1991). Strains of this pathotype follow a characteristic mechanism of epithelial cell penetration, lysis of the endocytic vacuole, intracellular

multiplication, movement through the cytoplasm and transverse dissemination to neighbouring enterocytes through their lateral cytoplasmic membranes (Parsot, 2005). Most EIEC pathogenesis results from plasmid-encoded type III secretion systems (Nataro and Kaper, 1998) although *Shigella* infection can also induce acute renal failure and haemolytic uraemic syndrome (HUS) (Houdouin et al., 2004).

Enteroaggregative *E. coli* is a recently defined diarrhoeal pathogen in multiple human population groups (Huang et al., 2006). There is no defined set of virulence elements associated with EAEC strains, and currently they are defined as *E. coli* lacking LT or ST toxins and adhering to human epithelial cells (Hep-2 cells) in a layered pattern (Harrington et al., 2006). EAEC pathogenesis involves adherence to the terminal ileum and colon mucosa mediated by aggregative adherence fimbriae (AAFs) resulting in characteristic mucus aggregates of bacteria and inflammation (Huang et al., 2006). Many strains harbour the plasmid encoded Pet toxin and can elaborate ShET1 enterotoxin, the former also present in most *Shigella flexneri* strains (Eslava et al., 1998; Henderson et al., 1999). Current data suggest that there is not a single set of virulence elements associated with all EAEC strains, and it has been proposed to reserve the term "typical EAEC" to strains harbouring the AggR protein, which controls a collection of genes that contribute to fimbrial biogenesis (Kaper et al., 2004).

Diffusely adherent *E. coli* is based on a phenotype that adheres to the surface of Hep-2 cells with a distinct pattern of dispersed adherence without aggregation. They have been implicated in diarrhoea of children

under 1 year of age in a limited number of reports (Le Bouguenec, 1999; Nataro and Kaper, 1998; Scaletsky et al., 2002) and evidence of DAEC pathogenicity is less clear than for EAEC (Huang et al., 2006; Robins-Browne and Hartland, 2002). Over 75% of the strains produce the F1845 fimbrial adhesin which binds to the brush border-associated decay-accelerating factor (DAF; CD55) as a receptor (Peiffer et al., 2000). Impairment of the microvilli associated hydrolases, sucrase-isomaltase and dipeptidylpeptidase IV following DAEC infection was suggested by the same group of workers as a possible pathological mechanism for DEAC induced diarrhoea (Peiffer et al., 2001).

Enterohaemorrhagic *E. coli* strains are characterised by inducing AE mucosal lesions and the production of Shiga toxin (Stx), also known as verocytotoxin (VT) (Frankel et al., 1998). Stx is the main bacterial virulence factor responsible for EHEC pathogenecity in humans (Nataro and Kaper, 1998). These cytotoxins are transported by neutrophils (Te Loo et al., 2001), and bind to the glycosphingolipid globotriaosylceramide receptor Gb3 which is found abundantly on human renal tissue (Lingwood, 2003). The Stx A subunit cleaves a single adenine residue from the 28S rRNA component of the host ribosome, producing inhibition of protein synthesis (Sandvig, 2001), and acts on endothelial cells of blood vessels predominantly in the intestinal submucosa and in the renal glomeruli (Nakao and Takeda, 2000). Endothelial heterogeneity in Shiga toxin receptors and responses may explain the particular involvement of the kidney (Obrig et al., 1993; Ohmi et al., 1998). Histopathologically, EHEC pathology is a thrombotic disorder, characterised by microvascular thrombi (Inward et al., 1997). This finding is supported by recent

research which suggested that prothrombin abnormalities might precede initial renal injury, and that renal insufficiency is secondary to fibrin thrombi deposition (Chandler et al., 2002; Tarr et al., 2005). The specific host mechanisms leading from Stx mediated injury of the vascular endothelium to thrombotic microangiopathy and renal injury remain to be elucidated.

1.1.2. *E. coli* O157:H7

Most of the worldwide disease caused by STEC is related to the *E. coli* O157:H7 strain, while approximately up to a third of the outbreaks are attributed to non-O157 isolates such as O26, O111, O128, and O103 (Bettelheim, 2000; Hussein and Omaye, 2003). Phylogenetically, enterohaemorrhagic *E. coli* O157:H7 is considered to have evolved from an EPEC O55:H7 strain by acquiring the Shiga toxin 2 (Stx2) genes, then the plasmid pO157 and shifting its antigen structure from O55 to O157 (Feng et al., 1998; Reid et al., 2000). Unlike the majority of other *E. coli* strains, *E. coli* O157:H7 does not usually ferment sorbitol, making sorbitol-MacConkey (SMAC) agar the medium of choice to isolate this organism in clinical and experimental specimens. Nevertheless, sorbitol-positive isolates of *E. coli* O157 have been described regularly in clinical and animal isolates and constitute a significant problem for diagnosis (Brunder et al., 2006). *E. coli* O157:H7 strains also harbour a pathogenicity island termed tellurite resistance- and adherence- island (TIA), which confers resistance to tellurite, resulting in the additional selective isolation medium cefixime-tellurite sorbitol-MacConkey agar (Tarr et al., 2000). Toxin production and O157 antigen can be detected by a variety of immunoassays and molecular methods (Lukinmaa et al.,

2004), but bacterial culture, demonstration of toxic effects on Vero cells, and genomic profiling by Pulsed Field Gel Electrophoresis (PFGE) are strongly recommended to facilitate typing and epidemiological investigations (Centers for Disease Control and Prevention, 2006; Gautom, 1997).

1.2. EHEC Virulence and colonisation factors

In EHEC evidence shows that virulence genes are inserted into diverse chromosomal loci among various serotypes, suggesting that each pathotype has emerged independently multiple times within *E. coli* by horizontal transmission (Wieler et al., 1997). EHEC strains are able to cause disease by the expression of multiple virulence factors (VFs) the most relevant of which are described in the following sections.

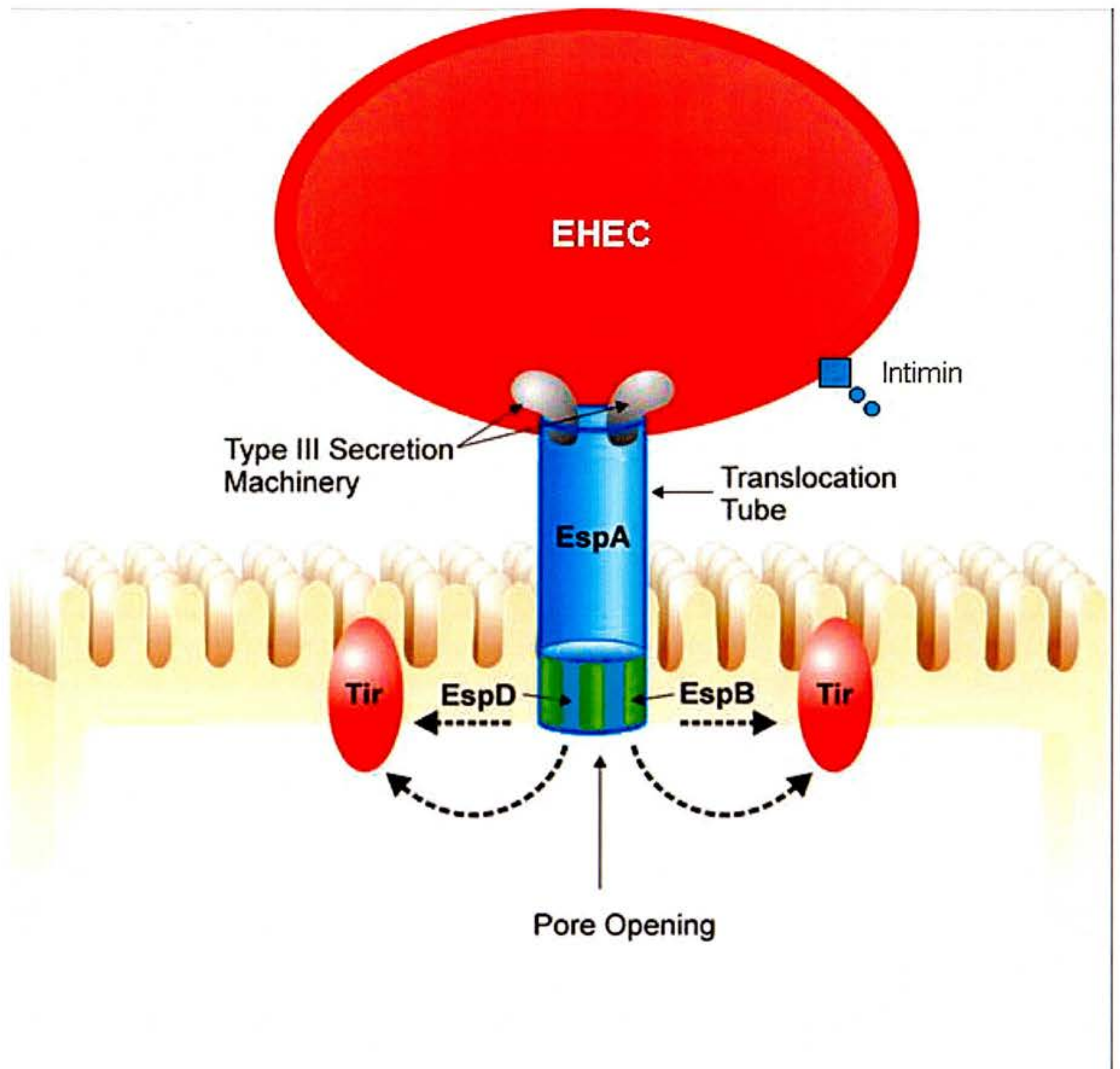
1.2.1. The Locus of Enterocyte Effacement

The Locus of Enterocyte Effacement (LEE) is a 43.3 kilobase pair (kb) chromosomal insertion with 54 open reading frames, that encodes for a group of factors required for the intimate attachment of the bacteria (Perna et al., 1998). The expression of these factors results in the hallmark of EPEC and EHEC infection: attaching and effacing lesions (AE) on the apical surfaces of host cells (Nataro and Kaper, 1998). This pathogenicity island has a lower guanidine-cytosine percentage (38.3%) than the rest of the bacterial genome which may indicate a different species origin (Elliott et al., 1998), and recent bioinformatics analysis has postulated lambda phage origins (Tobe et al., 2006). Originally described for EPEC strains, this morphologic alteration of the host cell is also

present when EHEC adheres to cell lines (Nicholls et al., 2000) and has also been reported in experimentally and naturally infected animals in various animals such as cattle (Hall et al., 1985; Moxley and Francis, 1986; Pospischil et al., 1987; Schoonderwoerd et al., 1988; Janke et al., 1989, 1990; Pearson et al., 1989; Dean-Nystrom et al., 1997; Naylor et al., 2005a), dogs (Janke et al., 1989), goats (Barlow et al., 2004), mice (Nagano et al., 2003), sheep (Wales et al., 2001a; Wales et al., 2005b), pigs (Helie et al., 1991; Neef et al., 1994; Higgins et al., 1997, Dean-Nystrom et al., 2000), rabbits (Peeters et al., 1984) and poultry (Fukui et al., 1995). Even though the resultant phenotype is similar, there are numerous differences in structure and regulatory inputs that suggest a tighter regulation of this large genetic element in EHEC than in EPEC strains (Spears et al., 2006). LEE is considered essential for EPEC/EHEC colonisation of the host (Nataro and Kaper, 1998), particularly in the bovine species. Signature-tagged mutagenesis and defined LEE deletions within O26 and O157 strains have shown the relevance of the LEE for colonisation in cattle (Dziva et al., 2004; Naylor et al., 2005b; van Diemen et al., 2005), and occasionally, atypical LEE negative EHEC strains have been isolated in human patients with severe disease (Elliott et al., 2001). A plasmid-encoded adhesin designated STEC autoagglutinating adhesin (Saa) has been identified in strains lacking the LEE, and this adhesin is now used routinely for characterisation of EHEC virulence factors (Nielsen and Andersen, 2003; Paton and Paton, 2002). Also, recent *in vitro* studies demonstrated the epithelial cell invasion of several EHEC-LEE negative strains, suggesting for these clones an alternative invasive mechanism to the customary membrane adherence (Luck et al., 2005).

Studies have shown that LEE genes are activated upon cell contact (Beltrametti et al., 1999; Wolff et al., 1998) and regulated by a quorum sensing mechanism through the *E. coli* regulator A (QseA) (Sperandio et al., 2002). The inducer has not been biochemichally identified yet, but is encoded by a member of the luxS gene family (Sperandio et al., 2003; Surette et al., 1999). The main consequence following LEE activation and protein secretion is the intimate attachment of the bacterium to the eukaryotic cell together with effacement of the enterocyte brush border leading to the formation of AE lesions. Through EspA, a TTSS nano-syringe, the bacterium injects its own receptor Tir, which binds to the bacteria outer membrane intimin, triggering the rearrangement of the epithelial cell actin and the formation of pedestal-like formations on the apical membrane of the mucosal epithelium (Vallance and Finlay, 2000; Figure 1-1). The ultimate benefits of this action for the bacteria may be varied; it may obtain access to cellular nutrients, enhance its persistence, or help the bacteria to avoid host immune responses.

Figure 1-1. Main proteins involved in the formation of the attaching-effacing lesion. Adapted from Vallance and Finlay, 2000.



The LEE contains 54 open reading frames organised into five operons, that encode components of a TTSS an organelle that spans the two membranes of the bacterium, and transfers proteins into the cytosol of the host cell (Mota et al., 2005). LEE operons 1 to 3, are highly conserved between EPEC and EHEC (Elliott et al., 1998; Jarvis et al., 1995), and encode several components of the TTSS, amongst others the translocated effector proteins EspH (Tu et al., 2003) and EspZ (Kanack et al., 2005).

LEE 4 genes encode for the regulator SepL (O'Connell et al., 2004) the needle EscF (Wilson et al., 2001), effectors such as EspF (Deng et al., 2005) and EspG (Ritchie and Waldor, 2005) and the translocators A, B and D. EspA forms an extension to the EscF that enters the host cell membrane via a pore formed by EspD and EspB (Yip and Strynadka, 2006). Regulated by its own promoter, the mitochondrial associated protein (MAP) is associated with filopodia formation (Kenny et al., 2002). LEE 5 encodes for effectors like the translocated intimin receptor (Tir) (Kenny, 2002) and the bacterial outer membrane adhesin intimin (Louie et al., 1993). So for all the LEE-encoded effectors translocated by the TTSS into the host cell, only TIR is indispensable for AE lesion formation (Spears et al., 2006).

1.2.2. Shiga toxins

Shiga toxins (Stx) are members of a family of plant and bacterial protein toxins that affect the translational machinery of the host cell, by removing a specific adenine base from the 28 S rRNA of the 60S ribosomal subunit of the parasitised eukaryotic cell (Endo et al., 1987). Stx are encoded in the genome of heterogenic lambdoid phages that when undergoing the lysogenic cycle, break the bacterial cell envelope causing the release of the toxin (Unkmeir and Schmidt, 2000). Stx are produced by EHEC isolated from human and non-human sources, *Shigella dysenteriae* type 1, and also occasionally, by *Citrobacter freundii, Enterobacter cloacae, Aeromononas hydrophila, Aeromononas caviae* and *Shigella flexneri* (Cherla et al., 2003; Sandvig, 2001). The Stx group contains two variants called Stx1 (also termed verocytotoxin 1: VT1 or Shiga-like toxin: SLT1) and Stx2 (VT2, SLT2). Stx1 is identical to shiga

toxin of *S. dysenteriae* type 1, whereas Stx2 is structurally and immunologically distinct (Fraser et al., 2004). These groups are composed of different variants or alleles, Stx1 type consists of Stx1, Stx1c, and Stx1d and the Stx2 group comprises Stx2, Stx2c, Stx2d, Stx2dactivatable, Stx2e, and Stx2f (Friedrich et al., 2002). The bacteria can harbour more than one Stx-encoding bacteriophage and therefore produce more than one type of Stx (Nakao and Takeda, 2000). The Stx type of the infecting strain can influence the prognosis of the infection in gnotobiotic pigs (Donohue-Rolfe et al., 2000) and people (Jure et al., 1998; Karch et al., 2006; Werber et al., 2003) with strains that produce Stx 2 perceived as more virulent, than bacteria that produce both Stx1 and Stx2, or Stx1. Some studies have indicated that among the different alleles stx2e can be considered to be of low pathogenecity, irrespective of the presence of other factors (Orth and Wurzner, 2006), while variants Stx2, Stx2c and Stx2d are associated with increased severity of disease and likelihood of progression to acute renal failure (Bielaszewska et al., 2006; Friedrich et al., 2002) in German patients. Although, there are reports that these associations have exceptions (Cimolai et al., 1994; Jelacic et al., 2002).

The only naturally occurring diseases in animals associated with Shiga toxin producing *E. coli* (STEC) are swollen head syndrome (SHS) in chickens, caused by Stx2y (Parreira and Yano, 1998; White et al., 1990) and oedema disease in piglets caused by Stx2e (Marques et al., 1987). In chickens the cytotoxic effect of the toxin has been described only *in vitro* (Salvadori et al., 2001). In pigs, the action of Stx2e toxin results on endothelial cells in small arteries and arterioles in the gut and brain

causing vascular damage in the gut and brain and death (Moxley, 2000). Nevertheless, serotyping and molecular profiling of isolates of *E. coli*-producing Stx2e from humans and pigs demonstrated that the strains that cause human disease are different from the strains that cause oedema disease in pigs (Sonntag et al., 2005), thus suggesting a non-porcine origin for the occurance of this toxin in humans.

Shiga toxins are composed of two structures; the A subunit carries the enzymatic activity, while the B subunit binds the toxin to the membrane receptor Gb3 in humans or Gb4 in pigs (DeGrandis et al., 1989; Jacewicz et al., 1986). The sites of pathology correlate with the presence of the toxin receptor glycolipid in humans, pigs and in the rabbit experimental model (Boyd et al., 1993; Lingwood, 1996; Zoja et al., 1992). In cattle, a major EHEC reservoir, Stx does not produce clinical disease despite the regular detection of shiga toxins in their faeces (Hyatt et al., 2001; Van Donkersgoed J. et al., 1999). Gb3 is present in both small and large intestinal mucosa (Hoey et al., 2002) but not in the vascular endothelium of cattle (Pruimboom-Brees et al., 2000). Interestingly work carried out with cell cultures of bovine intestinal epithelial cells exposed to Stx, showed the toxin localised in the lysosomes, in contrast with its restriction to endoplasmic reticulum in green monkey kidney Vero cells, a pathway that may inactivate Stx in the bovine species (Hoey et al., 2003). In the same work, no cytotoxicity was observed when bovine intestinal cells where challenged with doses of VT1 10,000 fold higher than the CD_{50} for Vero cells, confirming remarkable differences in cell sensitivity to the toxin. Spontaneous curation of Stx genes have been reported in bacteria shed in faeces during human infection (Mellmann et

al., 2005) and *in vitro* culture (Murase et al., 1999) . These findings may imply that the selective advantage that VT-encoding bacteriophages can provide to EHEC strains benefit the bacteria in other environments are still to be determined. Thus, the different pathology that shiga toxin induces in humans, pigs and rabbits, prevents the development of a suitable animal model, thus hampering the studies of human pathogenesis, which are now restricted to *in vitro* studies and clinico-pathological observations (Bielaszewska and Karch, 2005).

1.2.3. Other *E. coli* O157:H7 virulence factors

Of the shigatoxin-producing *E. coli* (STEC) the most studied is O157:H7 (Naylor et al., 2005a). Genetic analysis has shown that nearly 20% of the *E. coli* O157:H7 chromosome is not present in the benign laboratory strain K-12 MG1655 (Hayashi et al., 2001), and has probably been acquired through horizontal gene transfer (Reid et al., 2000). Such additional genes may be part of possible pathogenicity islands (PAIs), present in pathogenic strains but often not present in non-pathogenic clones, which like the LEE, carry virulence determinants and are inserted in excision tRNA loci and show a lower G:C content (Hacker et al., 2003). Sequencing of two epidemic strains, EDL 933 (Perna et al., 2001) and the Sakai strain (Hayashi et al., 2001) has led to the determination of several PAIs , some of these islands are described below.

Non-LEE encoded effectors: During the last two years the field of *E. coli* effectors has moved forward very quickly widening our knowledge of the pathological mechanisms observed during EHEC infection. These newly described lambdoid encoded effectors are translocated by the TTSS into

the eukaryotic cell and include Cif, EspI, TccP and EspJ (Garmendia et al., 2005). Cif blocks the cell cycle at the Gap2/Mitosis transition and induces the formation of stress fibres (Marches et al., 2003). EspI localises in the Golgi apparatus of the host cell and mutants of this gene show reduced colonisation efficacy in the *C. rodentium* mouse model (Mundy et al., 2004a). It has been found more commonly in patients than asymptomatic carriers (Mundy et al., 2004b). TccP is a protein that links Tir to the host cell actin cytoskeleton inducing actin polymerisation in a phosphorylation independent manner (Garmendia et al., 2004). EspJ is an effector not required for AE formation *in vivo*. However, experimental infections with EspJ mutant strains of *E. coli* O157:H7 in lambs and of *C. rodentium* in mice increased the persistence of the bacteria in the intestinal tract in these species (Dahan et al., 2005).

Plasmid pO157 is a 90Kb extrachromosomal element composed of four operons and 84 ORF's (Makino et al., 1998). The following pO157 determinants and proteins have been implicated in *E. coli* O157 pathogenesis. The espC-O gene encodes for a type II secretion system (SS), a highly specific apparatus that spans the periplasmic compartment, able to assemble a pilus (Schmidt et al., 1997). This type II SS is responsible for the secretion of toxins such as Stce, a C1-esterase inhibitor capable of suppressing the classic complement pathway in vitro (Grys et al., 2005; Lathem et al., 2004). The operon *hly* encodes for the synthesis and transport of enterohaemolysin, a pore-forming cytolysin, proven to be cytotoxic in bovine, ovine and human cells, (Bauer and Welch, 1996). Some workers have suggested that the role of this toxin is to provide a source of iron for the bacterium (Torres and Payne, 1997).

The *toxB* gene is highly conserved amongst *E. coli* O157 strains (Tozzoli et al., 2005) and influences the expression of the LEE resulting in reduced adherence of the bacteria *in vitro* (Tatsuno et al., 2000). Nevertheless, recent experiments with *toxB* mutants failed to show significant differences in their colonisation capacity in sheep and cattle (Stevens et al., 2004). (iv) The pO157 plasmid also encodes for other determinants that are considered putative virulence factors, including two enzymes, a catalase-peroxidase (KatP) (Brunder et al., 1996) considered part of the bacterial system of defence against oxidative stress (Farr and Kogoma, 1991; Spiro, 2006), and a serine-protease (EspP) that cleaves human coagulation factor V and to which there is an immune response in convalescent children (Brunder et al., 1997).

These plasmid determinants are heterogeneously distributed amongst O157 and other EHEC strains. The *hly* operon is present in almost all O157 strains, and is considered the best marker for detecting the presence of pO157. EspC-O, KatP and EspP are found in 50% of the clones (Caprioli et al., 2005). No correlation has been found between the presence of the genes and illness severity (Welinder-Olsson and Kaijser, 2005).

1.2.4. *E. coli* O157:H7 adhesins

Mechanisms of intimate cellular adhesion leading to AE formation have long been known. Despite this the initial adherence step before close intercellular contact is not well understood (Nataro and Kaper, 1998). A number of putative adhesin factors including type IV fimbriae, outer membrane proteins and flagellae have been described for *E. coli*

O157:H7. Analysis of the putative fimbrial operons from sequenced O157:H7 strains showed that several are likely to be cryptic with expression only demonstrated for three, one of which termed F9, has been reported to be needed for successful colonisation (Low et al., 2006b; van Diemen et al., 2005). *In vitro* studies and animal challenges with an F9 O157 mutant concluded that this adhesin is not involved in the rectal tropism (Low et al., 2006a). *E. coli* O157:H7 cannot express common type 1 fimbriae due to a conserved deletion in the *fimA* promoter region (Roe et al., 2001). *In vitro* studies of adherence to culture HeLa cells with mutagenised *E.coli* O157:H7 clones identified increased adherence in strains with increased expression of the outer membrane protein A (OmpA) (Torres and Kaper, 2003). This protein has also been involved in *E. coli* K-1 invasion of brain endothelial cells through its adhesion to the gp96 receptor in cases of neonatal meningitis (Prasadarao, 2002). *In vivo* work in cattle with isogenic deletion mutants to study their capacity to colonise the terminal rectum, found that the only virulence factors that had a significant impact in the bovine terminal rectum colonizing capacity of the bacteria were intimin, Tir and pO157, while Stx and haemolysin did not (Sheng et al., 2006a). A similar work carried out to study the role of flagellin, reported for both the mutant and the parent strain, a similar efficacy to colonise the bovine gastro intestinal tract (Dobbin et al., 2006). While Tir and intimin are pivotal in the intimate attachment of the bacteria to the mucosal epithelium, the function of pO157 in colonisation is not well understood.

1.3. Overview EHEC in humans

Our current understanding of the epidemiology of EHEC strains in humans is constrained by two issues. One is the lack of a clear proportional relationship between the prevalent EHEC animal strains and those that cause more frequent disease in humans (Kuhnert et al., 2005; Pearce et al., 2006; Roldgaard et al., 2004). The other, is that the incidence of non-O157 human infections is underestimated by detection costs and isolation complexity associated with the numerous potential serotypes involved (Fairbrother and Nadeau, 2006; Johnson et al., 2006). Both restrictions are difficult to resolve given the complexity of reservoirs and transmission modes of this pathotype and the number of strains involved.

There are over 250 different O serotypes, of which more than one hundred have been associated with human disease ranging from diarrhoea to HUS (Johnson et al., 2006) (un updated list can be accessed at: http://www.microbionet.com.au/vtectable.htm). While strain O157:H7 is the predominant EHEC serotype in the United States, Canada and the United Kingdom and the clone most commonly associated with HUS (Nataro and Kaper, 1998), non-O157 serotypes may account for up to 20-50% of the cases in the United States (Brooks et al., 2005) and United Kingdom (Kleanthous et al., 1990). In contrast, other countries around the world report higher incidences of non-O157 induced human disease such as Denmark (75% of STEC associated diarrhoeas originated from non-O157 cases), Spain (78%), Finland (53%), Czech Republic (57%), Belgium (63%), Chile (63%) and Australia (69%)

(Johnson et al., 2006) Singularly, New Zealand has never reported the isolation of *E. coli* O157 from clinical isolates (Brooks et al., 1997) or meat (Bennett and Bettelheim, 2002). Different regulations between countries on statutory surveillance, notification and detection methods precludes meaningful comparisons of these incidence figures.

Amongst the most common non-O157 serotypes causing human disease in Europe are O26:H11/HNM, O103:H2, O111:HNM, and O145:HNM (World Health Organization, 1998) with O26:H11 reported as the most common non-O157 isolate from HUS cases (Gerber et al., 2002; Misselwitz et al., 2003). It is commonly accepted that due to the availability of selective isolation media, laboratory detection favours *E. coli* O157 (Goldwater and Bettelheim, 1998; Thorpe et al., 2002). The importance of adequate strain identification was demonstrated during an outbreak in Washington State where rapid outbreak recognition and control prevented an estimated 800 cases (Bell et al., 1994). To achieve a complete response international networks such as Med-Vet-Net and Enter-Net in Europe, Australia and Japan, and the Food-borne Diseases Surveillance Network (FoodNet) of the Centre for Disease Control in the USA, play an important role in harmonisation of protocols, rapid reporting and promoting concerted programmes for research and surveillance.

Since the first association between *E. coli* O157:H7 and thrombotic microangiopathy (Karmali et al., 1983; Riley et al., 1983), the incidence reported by the Department of Environment, Food and Rural Affairs in the UK and the Centers for Disease Control and Prevention in the USA of

the disease attributable to this strain in particular, and to the other EHEC serogroups, has stabilised to 1.6 and 2.5 cases per 100,000 population in the UK and USA since the years 1995 and 2001 respectively (Brooks et al., 2005; Department for Environment, Food and Rural Affairs, 2005; Rangel et al., 2005). The incidence in Scotland has also stabilised in the last decade, although the number of human cases stabilised around 4.0 per 100,000 population, twice the annual incidence rate for England and Wales (Locking et al., 2006). These overall trends suggest that the systematic preventive approach to food hygiene established by Hazard Analysis and Critical Control Points (HACCP) and detection systems such Health Protection Scotland and PulseNet in the USA for the molecular subtyping of EHEC in humans, food, water, and the environment, are starting to be effective (Gerner-Smidt et al., 2005).

Enterohaemorrhagic *E. coli* was first recognised as a cause of illness in 1982 (Riley et al., 1983). It is a zoonotic disease and ruminants are its principal reservoir, although EHEC strains can be found in numerous animal species (Naylor et al., 2005a) including asymptomatic human carriers (Stephan and Untermann, 1999; Wilson et al., 1998). Infection is restricted to humans who commonly develop afebrile haemorrhagic colitis and HUS which is characterised by renal failure, thrombocytopenia and microangiopathic haemolytic anaemia (Amirlak and Amirlak, 2006). A limited number of HUS cases have been associated with acute urinary infection with EHEC strains (Starr et al., 1998). Frequent extrarenal abnormalities include neurological damage, colonic necrosis, myocardial dysfunction and pancreatitis (Brandt et al., 1994; Gerber et al., 2002; Taylor et al., 1986). Necropsy and histological studies

have also reported that the kidney, followed by the large bowel, the central nervous system, the heart and the pancreas are the organs more affected, thus corroborating the clinical findings (Gallo and Gianantonio, 1995).

1.3.1. *E. coli* O157:H7 in humans

E. coli O157:H7 is one of the most notorious serotypes amongst verocytotoxigenic *E. coli*. It is associated with serious human disease and infection can result not only in diarrhoea but haemolytic-uraemic syndrome and death (Karch et al., 2005). *E. coli* O157:H7 was first recognised as a cause of illness in 1982 during an outbreak of severe bloody diarrhoea, where infection was traced to the consumption of contaminated hamburgers (Centers for Disease Control and Prevention, 1982). Since the first description of this illness, infections have been reported from more than 30 countries on six continents (Thorns, 2000).

During the initial years of reporting, *E. coli* O157:H7 outbreaks were predominantly foodborne and often involved insufficiently cooked, ground beef contaminated during the slaughter process (Slutsker et al., 1998). Raised awareness and increased surveillance led to an increase in clinical cases and to the identification of diverse contamination sources. *E. coli* O157 excreted in animal faeces and occasionally spread by manure fertilisation can also contaminate farm products, such as unpasteurised apple juice (Hilborn et al., 2000), pasteurised (Goh et al., 2002) and unpasteurised milk (Liptakova et al., 2004), alfalfa sprouts (Breuer et al., 2001), as well as soil (Mukherjee et al., 2006) and water (Friedman et al., 1999). Transmission may also occur by person to

person spread (Willshaw et al., 2001) including asymptomatic carriers (O'Brien et al., 2001a; Strachan et al., 2006), airborne dispersion (Varma et al. 2003), and has occurred in laboratories (Public Health Laboratory Service, 1996). Infectious dose is low and it has been estimated that a few hundred bacteria may produce infection in humans and cattle (Besser et al., 2001; Tuttle et al., 1999).

The majority of human cases are sporadic and the source of infection is generally unknown (Naylor et al., 2005b), although most major outbreaks in the USA and UK can be linked to *E. coli* O157 tainted food (O'Brien et al., 2001b; Rangel et al., 2005). In Scotland, although one of the deadliest outbreaks in the world occurred in Lanarkshire in 1996, and was linked to contaminated meat (Cowden et al., 2001), the majority of cases of *E. coli* O157 now appear as sporadic cases (Evans et al., 2000; Locking et al., 2006). The main risk factor identified in several studies suggest that these scattered cases are the result of environmental contact with animal faeces (Coia et al., 1998; Locking et al., 2006; O'Brien et al., 2001a) through water, soil and direct contact with animals (Solecki et al., 2007; Strachan et al., 2006). Therefore, an important issue for prevention of human infection through either food or contaminated environment must be to understand and control *E. coli* O157 carriage in animals.

1.4. Overview EHEC in animals

The common features that characterise the heterogeneous EHEC pathotype are lack of specific host or niche and ubiquitous distribution

in ruminants. EHEC strains have been isolated consistently in countries from all continents, including Europe (Ammon, 1997; European Food Safety Agency, 2007), USA (Centers for Disease Control and Prevention, 2007; Gerner-Smidt et al., 2006), Argentina and Brazil (Guth et al., 2003), Africa (Raji et al., 2006), India (Wani et al., 2003), Thailand (Panutdaporn et al., 2004), Australia (Cobbold and Desmarchelier, 2001) and China (Zhou et al., 2002). Ruminants are considered the main reservoir for EHEC strains (Kaper et al., 2004; Naylor et al., 2005a), although EHEC strains have been detected in a variety of animals, such as; pigs (Fratamico, et al. 2004), wild deer (Asakura et al., 1998), pigeons, gulls and broilers (Kobayashi et al., 2002), rabbits (Garcia and Fox, 2003), and zoo ungulates (Leotta et al., 2006). *E. coli* O157 has also been found in a similar wide range of animal species including pigs (Aktan et al., 2004), deer (Asakura et al., 1998), wild birds, rodents (Nielsen et al., 2004a), chickens (La Ragione et al., 2005), ducks (Leclercq and Mahillon, 2003), wild rabbits (Scaife et al., 2006), healthy and sick commercial rabbits (Blanco et al., 1996), horses (Chapman, 2000), earthworms (Williams et al., 2006), slugs (Sproston et al., 2006), and houseflies (Kobayashi et al., 1999). Although there is circumstantial evidence of transmission of *E. coli* O157 from wild rabbits (Scaife et al., 2006) and birds (Ejidokun et al., 2006) to humans, most authors agree that these species act predominantly as mechanical vectors or as reservoirs for ruminant infection (Alam and Zurek, 2004; Cizek et al., 1999; LeJeune et al., 2004; Rice et al., 1995; Sanderson et al., 2006).

1.4.1. Cattle as reservoirs

Over 400 EHEC strains have been isolated from cattle (Gyles, 2007) many of which are found simultaneously in a herd. One major study detected up to 170 different EHEC strains circulating in a single cohort of 49 healthy cattle (Shaw et al., 2004). Several studies have described within herd epidemiology and shedding of EHEC strains. In a longitudinal study based on 12 strains, environmental transmission accounted for 60% of all infections, while calf-to-calf transmission explained the other 40%. Shedding was sporadic with the same strain detected in the same animal weeks apart (Liu et al., 2005; Pearce et al., 2004a). Duration for individual infection was established at 3-5 days on average depending on the strain (Liu et al., 2005). Remarkably, *E. coli* O157 strains can persist on farms for up to two years (Shere et al., 1998). These findings confirm the relevance of cattle as a major reservoir of EHEC strains, many of which coexist within the same herd for long periods. Infection in cattle is usually short and can recur indicating either reinfection or shedding periods with bacterial numbers too low to be detected.

The amount of research published during the last thirty years on *E. coli* O157:H7 has promoted this serotype to the *de facto* group model. In the bovine species, *E. coli* O157:H7 estimates from field studies report great variability in point prevalence. Values observed range from 0% to 57% (Callaway et al., 2005; Khaitsa et al., 2006; Meyer-Broseta et al., 2001; Vidovic and Korber, 2006), and excretion is more prevalent among calves aged 3–16 months (Garber et al., 1995; Paiba et al., 2003; Rugbjerg et al., 2003). Estimates from slaughterhouses reflect similar wide

prevalence values from 3% to up to 37.5% (Fukushima and Seki, 2004). Similar prevalence rates of up to 58% have been reported for non-O157 STEC strains (Hussein, 2006).

These prevalence surveys are based on cross sectional studies, which describe carriage status at a particular point in time, and can only provide a static view on the infectious process dynamics. In contrast, longitudinal analysis involves observations of the same individuals and farms over long periods, recording the temporal order of events and is more informative to study transmission dynamics than cross sectional studies, because they can track changes at group and individual level. Several studies involving cohort, or panel, cross-sectional studies have been published (Cho et al., 2006; Dopfer et al., 2006; Ezawa et al., 2004; Sanderson et al., 2006; Schouten et al., 2005). These works report that faecal shedding of *E. coli* O157:H7 is limited to a few individuals and farms separated by periods of low prevalence or absence of infection. Mathematical analysis of a large two year cross-sectional survey of 481 farms showed that, the best theoretical distribution to reproduce *E. coli* O157 prevalence values, incorporated a subset of highly infectious animals. These animals with a high level of carriage are about 20% of the colonised population and account for 80% of the transmission (Matthews et al., 2006a). Targeted measures aimed at preventing infection in the 5% of individuals with the highest overall infectiousness would reduce the number of secondary cases a single infected case will cause, reducing the reproduction ratio [or R_O) below one, therefore eliminating the infection from the herd (Matthews et al., 2006b; Woolhouse et al., 1997). Significantly, a cohort study carried out on naturally infected animals

reported a small proportion of the infected animals becoming persistent high shedders (*E. coli* O157:H7 bacterial counts above 10^3 colonies forming unit per gram of faeces, CFU g^{-1}) for at least fifteen days (Cobbold et al., 2007; Robinson et al., 2004). Slaughterhouse studies have, to a degree, confirmed these predictions by identifying between 3.7% (Low et al., 2005c) to 1.86% (Omisakin et al., 2003) of animals as high shedders. It must be noted that these abattoir studies lack longitudinal data to allow discrimination between different stages of animal infection (variation within animals) and genuine animal variation in the ability to carry and shed the bacteria (variation between animals).

E. coli O157 seasonal shedding variability remains controversial, with some studies reporting a higher prevalence in summer and autumn (Lahti et al., 2003; Tutenel et al., 2002), while others described a higher winter prevalence (Ogden et al., 2004; Shere et al., 1998) and some failing to detect such seasonal trend (Alam and Zurek, 2006; Dopfer et al., 2006; Mechie et al., 1997).

1.4.2. Other ruminants

EHEC prevalence surveys in sheep and goats offer similar ranges to those reported for the bovine species (Beutin et al., 1993; Fegan and Desmarchelier, 1999; Urdahl et al., 2001; Vettorato et al., 2003; Zweifel et al., 2004) and show that small ruminants can be considered a significant public health hazard. Most ovine strains are host specific (Beutin et al., 1997; Djordjevic et al., 2004) and a number of them have been linked to human disease (Bettelheim et al., 2000b; Orden et al., 2003). EHEC strains are three to four fold more prevalent in sheep and

goats than in cattle in Denmark (Beutin et al., 1993)and Serbia (Cobeljic et al., 2005), while sheep are considered greater EHEC spreaders than cattle in Australia (Gyles, 2007) and the main source of *E. coli* O157:H7 environmental contamination in Scotland (Strachan et al., 2005).

Sheep are a known reservoir of *E. coli* O157 and this strain is better adapted to persist in sheep than are other common pathotypes of *E. coli* (Cornick et al., 2000). Sheep can shed *E. coli* O157:H7 in amounts exceeding 10^3 CFU g^{-1} (the threshold for cattle to be considered a super shedder) (Cornick et al., 2000; Matthews et al., 2006b; Ogden et al., 2005). Bacterial numbers have been reported highest in faeces, and as in cattle, rectal localisation has been described (Grauke et al., 2002; Wales et al., 2001a).

1.4.3. Pigs

Pigs can also act as asymptomatic carriers of EHEC strains, including O157 (Kaufmann et al., 2006), O26 (Leomil et al., 2005), O111 (Notario et al., 2000), O103 (Aktan et al., 2004), and O145 (Krause et al., 2005). Pigs have been found to shed *E. coli* O157:H7 up to 10^4 CFU g^{-1} (Cornick and Helgerson, 2004) with a reported persistence of up to two months in experimentally challenged pigs (Booher et al., 2002), and several studies have identified pigs as a risk factor for herds being STEC O157 positive (Eriksson et al., 2005; Schouten et al., 2004). Faecal carriage of strain O157 in pigs at slaughter ranges from 0.2% to 2% in Europe (Bonardi et al., 2003; Chapman et al., 1997; Heuvelink et al., 1999; Johnsen et al., 2001), 2% in Japan (Nakazawa and Akiba, 1999) and USA (Feder et al., 2003), to 3.3% in China (Zhou et al., 2002) and up to 10% in Chile (Borie

et al., 1997). Prevalence reports on farms ranges from 0.1% in Sweden (Eriksson et al., 2003) to 2.1% in Mexico (Callaway et al., 2004) and up to 8.9% in some states of the USA (Doane et al., 2007). To the present date there are only two reports, one in the USA and one in Italy of *E. coli* O157:H7 human outbreaks being associated with pigs (Conedera et al., 2007; Jay et al., 2007; Solecki et al., 2007). In addition, a clonal relationship by colony blot hybridisation and PFGE has been reported among O157 isolates obtained from HUS cases and pigs in Chile (Rios et al., 1999), while a recent study in Switzerland isolated three serotypes previously reported in human STEC causing HUS (O9:H-, O26:H-, and O103:H2) in faeces from slaughtered pigs (Kaufmann et al., 2006). Therefore, it may be possible for an undetermined number of human cases to have originated from contact with pig faeces, and risk may be related to geographical differences in pig husbandry and slaughtering practices, or to lack of adequate source identification.

1.5. *E. coli* O157:H7 bovine colonisation

It is commonly agreed that *E. coli* O157 is not pathogenic for cattle over 1 week of age (Dean-Nystrom et al., 1999; Garber et al., 1995), although it may cause diarrhoea in experimentally challenged newborn (<36 h old) colostrum deprived calves (Brown et al., 1997; Cray Jr. and Moon, 1995) and at <12 h old can develop severe, often fatal, diarrhoea accompanied by AE lesions (Dean-Nystrom et al., 1997). The resistance of calves and older animals to *E. coli* O157:H7 infection may be explained by the lack of vascular receptors for Shiga toxins. This absence of Stx specific Gb3 receptors makes the bovine species inherently insensitive to the toxin

(Pruimboom-Brees et al., 2000). Thus the general consensus is that in naturally colonised cattle *E. coli* O157:H7 behaves as a commensal, a type of symbiotic relationship in which the bacteria benefits from the association while cattle are not affected (Cray, Jr. and Moon, 1995; Pruimboom-Brees et al., 2000; Wray et al., 2000).

1.5.1. Site of colonisation

Initial experimental challenges in weaned calves reported a variety of gastrointestinal colonisation sites. An early study carried out in experimentally challenged calves and adult cattle detected consistently higher *E. coli* O157:H7 numbers in the contents of the large intestine and faeces (Cray, Jr. and Moon, 1995). In contrast, another study reported the bacteria predominantly in the gastrointestinal contents of the rumen, with comparatively lower numbers obtained from cultures of tissue washings (Brown et al., 1997). The finding lead the authors to suggest that the predominant localisation of *E. coli* O157:H7 was in the contents of the forestomachs rather than on the mucosal surfaces of the large intestine. A more recent study in adult cattle, confirmed the inconsistent culture of *E. coli* O157:H7 from gastrointestinal tissues, and reported the bacteria to be most prevalent in the lower gastrointestinal digesta, specifically the caecum, colon, and faeces. In consequence, the authors proposed the colon contents as the main site for *E. coli* O157:H7 persistence and proliferation (Grauke et al., 2002). Interestingly, a histopathogical study made in high shedding animals reported the rectum as the major site of colonisation followed by the colon (Dean-Nystrom et al., 1999). In summary, two of these studies report a predominantly *E. coli* O157:H7 colonic localisation and found the highest

numbers in the faeces. All three, except the study carried out on high shedders, failed to demonstrate any evidence of mucosal colonisation or AE lesions in infected animals.

Recently the terminal rectum was identified as the principal site for *E. coli* O157:H7 colonisation in cattle and independent of tissue bacterial loads (Naylor et al., 2003a). This important finding has been fully corroborated by similar studies carried out on feedlot cattle (Cobbold et al., 2007; Lim et al., 2006), and by evidence that direct application to the rectum was more successful in achieving long-term colonisation of calves than oral challenge (Sheng et al., 2004). Support for the importance of terminal rectum colonisation also comes from a recent abattoir study in naturally colonised animals where the terminal rectum was colonised in 15 out of 16 *E. coli* O157 positive animals (Low et al., 2005). Nevertheless, it is apparent that the colonisation of other sites situated higher up in the intestinal tract may occur, and their relevance for sustaining infection has not been studied previously.

1.5.2. Histopathology in cattle intestine

The distinguishing histopathological feature of *E. coli* O157:H7 infection in cattle is the attaching and effacing (AE) lesions, characterised by elimination of the microvilli and intimate enterocyte attachment (Dean-Nystrom et al., 1999). This characteristic alteration of the enterocyte morphology has been reported to be accompanied by an increase in neutrophils and eosinophils in the lamina propria of the large intestine (Woodward et al., 1999), colon and caecum (Dean-Nystrom et al., 1999), gall bladder (Stoffregen et al., 2004), and sections of ligated ileal loops

(Sandhu and Gyles, 2002). This type of inflammatory reaction has been described in the intestinal tract exclusively for experimental infections of gnotobiotic, neonatal or immunosuppressed calves. In weaned calves an eosinophilic infiltrate has been reported only for the perianal skin and in areas where AE lesions were present (Pohlenz and Dean-Nystrom, 2004). All these studies are based on qualitative estimations, with no internal or external controls. No detailed pathological studies have been carried out in the terminal rectum, the main area of *E. coli* O157:H7 colonisation in the bovine species beyond the original study performed by Naylor *et al.* (2003).

1.5.3. Immune responses to *E. coli* O157:H7

Serological responses against *E. coli* O157:H7 bovine infections have been reported in the scientific literature. A seroprevalence study in the USA, reported the prevalence of anti O157 antibodies at 83% in calves and 100% in herds (Laegreid et al., 1999). Increased serum levels of IgG but not IgM were described for three out of six experimentally challenged calves. In contrast, the same study did not detect raised IgG titres in three dry cows (Wray et al., 2000). In another study, increased serological IgG responses against *E. coli* O157 LPS and shigatoxin 2 were detected following experimental infection of four calves and six steers, but in this work the serological responses were not correlated with elimination of infection. In a rechallenge study carried out in four weaned calves, reinfection with the same strain resulted in lower levels of shedding (Cray, Jr. and Moon, 1995). Neutralizing anti-shigatoxin IgG1 antibodies were present in 84.0% (189/225) of colostrum samples and in 93% (37/40) of sera from heifers from randomly chosen cows in Germany

(Pirro et al. 1995). Colostrum administration from dams with antibody titres against *E. coli* O157 in sera effectively elevated serum antibodies to *E. coli* O157 LPS in neonatal calves, even though no protection assays were undertaken in this study (Widiasih et al., 2004a). Overall, these results support the theory that in cattle there are serological IgG responses against *E. coli* O157:H7 colonisation and the latter work suggests that STEC exposure of cows is widespread. To date, studies have not been undertaken to detect mucosal immune responses to *E. coli* O157:H7 gastrointestinal colonisation.

1.6. Control strategies in the animal reservoir

The remarkable variety and high prevalence in ruminants of EHEC strains makes attempting to reduce their overall population an unattainable goal, thus the efforts to protect public health against these human pathogens should concentrate on blocking transmission pathways by improving food and water safety (Gannon, 1999; World Health Organization, 2001). It is possible that targeted interventions in the animal reservoir against *E. coli* O157:H7 could significantly reduce the number of human cases, as most outbreaks are linked to both this strain and ruminant sources (O'Brien et al., 2001a; Rangel et al., 2005).

A number of possible interventions including the use of antibiotics, vaccination, bacteriophages, competitive exclusion, water hygiene, changing dietary practices and good animal management can potentially reduce the numbers of pathogenic bacteria that enter the food chain and environment and will be briefly described in the following sections.

1.6.1. Direct application of antibactericidials to the terminal rectum

Following the recent determination of the terminal rectum as the principal site of *E. coli* O157 in cattle, two different treatments based on the principle of the direct application of antibactericidals to this area have been tested in cattle: a lytic bacteriophage and the antiseptic Chlorhexidine. In both cases, the treatment initially reduced bacterial levels but did not prevent ensuing *E. coli* O157:H7 carriage. Although none of the treatments consistently cleared the bacteria from cattle, both were successful in reducing the shedding levels from 10^4 – 10^5 CFU g^{-1} of *E. coli* O157:H7 to below 10^3 log values on day 1 after treatment (Naylor et al., 2007; Sheng et al., 2006b). The phage was administered a further three times but failed to produce any further reductions in shedding. Several reasons could explain the partial success of the antibactericidals including; inability of the treatment to eliminate all the bacteria, recolonisation of the terminal rectum from bacteria originating from other sites situated higher up in the gastrointestinal tract, or bacteria shedding from these sites. The direct rectal application of these treatments had a beneficial therapeutic effect by eliminating the animals considered as high shedders. Further improvements in product choice or administration methods may better the current results, and offer a viable control option to maintain "*E.coli* O157:H7 clean" herds. Alternatively, the use of these methods prior to transport could limit the transmission of *E.coli* O157:H7 to other animals during transport or lairage.

1.6.2. Vaccination

A successful vaccine should induce reductions in shedding to levels so that the on farm transmission of the pathogen ceases. The exclusively gastrointestinal location of *E. coli* O157:H7, requires a vaccine to induce specific secretory immunoglobulin A responses as well as high colostral IgG1 responses to prevent intestinal colonisation of both adult and newborn calves (Stevens et al., 2002). Several groups are currently developing *E. coli* O157:H7 vaccines for cattle. In an interesting study carried out by the National Institute of Animal Health, in Japan, intranasal administration of recombinant intimin mixed with *E. coli* heat-labile enterotoxin as adjuvant, induced elevated IgA-specific antibody in the nasal secretion and saliva of calves and elevated IgG1-specific antibody level against the intimin in the sera and colostrum of cows (Yokomizo et al., 2002). Although no trial was conducted to asses the protection of this vaccine, several studies suggest that intimin is an important antigen for any vaccine formulation. Immunisation of pregnant swine with purified *E. coli* O157:H7 intimin induced serum and colostrum antibodies in the dams, and an increase in resistance to colonisation and intestinal damage in suckling piglets (Dean-Nystrom et al., 2002). Intimin-specific immune responses prevented bacterial colonisation of the mouse gut by *Citrobacter rodentium,* a related enteropathogen used as EHEC and EPEC models of intestinal colonisation (Ghaem-Maghami et al., 2001). A recent vaccine trial based on secreted proteins (Tir, EspA, B, D and P) of the Type III system used by *E. coli* O157:H7 for intestinal colonisation failed to reduce significantly the numbers of bacteria shed on faeces on 218 pens of feedlot cattle in Canada (Potter et al., 2004; Van Donkersgoed J. et al., 2005).

1.6.3. Other treatments

A number of studies have proposed the use of probiotics and bacteriophages to control *E.coli* O157:H7 in ruminants. Neomycin administration has been reported to decrease faecal shedding in cattle (Edrington et al., 2003; Ranson et al., 2003), although given the current concern on the spread of antibiotic resistance, it is unlikely that the prophylactic use of antibiotics would ever be approved. Various *E.coli* O157:H7 specific bacteriophages have been isolated by different researchers, but all showed low *in vivo* effectiveness when administered orally (Bach et al., 2002; Kudva et al., 1999; Tanji et al., 2004). Competitive inhibition with other bacteria is a well established technique for reducing *Salmonella* species colonisation in poultry and recently approved by the FDA for its use (Nisbet, 2002). At least two studies have been published in which different bacterial isolates that were determined to inhibit *E.coli* O157:H7 *in vitro* were given orally to calves and reduced the levels and duration of carriage (Ohya et al., 2000; Zhao et al., 1998). These studies were carried out in a limited number of animals and larger field studies are needed to prove a real impact of probiotic bacteria on the ecology of *E.coli* O157:H7 in the cattle population.

1.6.4. Diet and fasting

There are conflicting reports regarding the effects of changes in the diet on faecal shedding of *E. coli* O157:H7. While some studies suggest that grain feed often used to maximise growth efficiency, can increase shedding ((Dargatz et al., 1997; Diez-Gonzalez et al., 1998), others found no difference in amount or duration of shedding between animals fed

grain or forage diets (Buchko et al., 2000; Hovde et al., 1999; Magnuson et al., 2000) and the effect of forage feeding remains an area of contention amongst researchers (Callaway et al., 2003).

Fasting cattle before abattoir transport is a common practice to reduce soiling of the hides, which are considered the principal source of carcass contamination (Gannon, 1999). To our knowledge, no study has detected any relation between fasting and *E. coli* O157:H7 shedding in cattle (Brown et al., 1997; Cray Jr. et al., 1998; Harmon et al., 1999) or sheep (Kudva et al., 1997)

1.6.5. Management

Identified environmental farm factors that influence *E. coli* O157:H7 transmission in cattle include manure processing and handling practices (Kudva et al., 1998; Rasmussen and Casey, 2001) and contamination of water troughs, where the bacteria can remain infectious for ten months (LeJeune et al., 2001). Tainting of water tanks has been consistently associated with *E. coli* O157:H7 presence in the herd by different researchers (Meyer-Broseta et al., 2001; Smith et al., 2005; Van Donkersgoed J., et al. 2001).

1.7. Conclusion and aims

E. coli O157:H7 remains a substantial risk to public health despite considerable research and public health efforts since its first identification as a human pathogen in 1982. The illness this organism causes is serious and sometimes fatal, and largely untreatable. In

consequence, reducing the morbidity and mortality depends greatly on preventing infections. Cattle are considered a primary reservoir of EHEC bacteria, and particularly, of *E. coli* O157:H7 one of the most pathogenic and prevalent of the EHEC strains. Therefore, an important issue for prevention of human infection must be to understand and control *E. coli* O157:H7 carriage in the bovine host.

The aims of this study is to understand the carriage and persistence of *E. coli* O157:H7 in cattle. At the beginning of this project, it was clear that *E. coli* O157:H7 colonisation is associated with the terminal rectal mucosa. However, the reasons for such a tropism, the relevance of other potential colonisation sites in the gastrointestinal tract, the attachment mechanisms operating, and details of host-pathogen interactions were unknown.

The objectives of this study are (i) to confirm the terminal tropism of *E. coli* O157:H7 in cattle in a larger number of animals; (ii) to study the shedding patterns and bacterial distribution in both faeces and gastrointestinal contents; (iii) to determine the importance if any of other sites in the gastrointestinal tract for *E. coli* O157:H7 carriage; (iv) to confirm epithelial attachment at the rectum by transmission electron microscopy; (v) to define the mechanisms of adherence to the gastrointestinal epithelium; (vi) to determine if colonisation of the terminal rectum in cattle induced pathology in terms of microscopic and ultrastructural change to the epithelium; (vii) to determine if colonisation of the terminal rectum induced mucosal innate or acquired immune

responses at the site of infection and (viii) to define the major bacterial immunogenic mucosal antigens.

Chapter 2

Material and Methods

2.1 Bacterial strains

The challenge strain of *E. coli* O157:H7 was ZAP 198 isolated from a human patient in Washington State (USA) were cattle were establish as the source of the outbreak. and used previously in experimental studies (Naylor et al., 2003b). ZAP 198 has been naturally cured of the shiga-toxin (Stx2) carrying bacteriophage and the strain was selected for spontaneous resistance to nalidixic acid to facilitate recovery from faeces and tissues. ZAP 198 possess genes for enterohemolysin, intimin-γ, EspA and EspB. For preliminary examination of adaptative responses whole cell extracts of *E. coli* K12 MG1655 ZAP 185 (Blattner et al., 1997) and *E. coli* O26 ZAP 194 (Pearce et al., 2006) were used as controls in Western blots. The protein content was 5 mg $gram^{-1}$, determined with BCA Protein Assay Kit (Pierce, USA) according to the manufacturer's instructions. The colorimetric absorbance was read at wavelength of 562 nm in a Dynex Revelation 4.02 Spectrophotometer (Dynex Technologies). Their main characteristics and sources are listed in Table 2-1.

Table 2-1. Bacterial strains

Strain	Description	Source/Reference
ZAP 185	*E. coli* K12 MG1655	(Blattner et al. 1997)
ZAP 194	*E. coli* O26	(Pearce et al. 2006)
ZAP 198	*E. coli* O157:H7 VT1– VT2–	(Ostroff et al. 1990)
ZAP 1187	*E. coli* O157:H7 ompC negative	This study

2.2 Animal groups and bacterial challenges

Experimental procedures (authorised by Home Office licence 60/3179) were carried out at the Moredun Research Institute. Prior to experimental challenge faecal samples were taken at least twice from each calf and confirmed negative for *E. coli* O157:H7 by immunomagnetic separation (IMS). The challenge *E. coli* O157:H7 ZAP 198 was grown overnight in Luria Bertani (LB) broth at 37°C, with aeration, and diluted in sterile PBS to achieve an inoculum of 10^9 CFU of *E. coli* O157:H7 per animal in a total volume of 10 ml. The 10 ml oral inoculum was administered into the rumen to the calves via stomach tube and washed down with 500 ml sterile PBS, or by direct administration through a cannula placed on the dorsal rumen and sutured to hide of dorsolateral abdominal wall in six calves. Rectal challenge was carried out by loading a large cotton swab, consisting of 2 cm long wad, with the challenge inoculum followed by repeated direct application to the mucosal surface of the recto-anal junction until exhaustion of the 10 ml inoculum. Five groups of weaned calves were used for the various aspects of the work described in this thesis.

Group 1. Fifty-four, weaned, Holstein-cross calves were reared conventionally on a farm. The animals were aged between 8 to 14 weeks on arrival and were then penned individually for the study. Calves were fed concentrate twice daily and had access to *ad lib* hay and water. Feed, water and bedding were provided separately for each animal to minimise cross contamination and each was haltered to reduce the opportunity for faecal-oral transmission. On a single occasion 10 ml of inoculum was

administered by three different routes, 24 calves were challenged via the rectal route, 6 intraruminally through a cannula placed through the paralumbar fossa on the dorsal rumen, and 24 by oral administration via stomach tube on a single occasion. Initial screening of newly arrived calves by IMS detected one animal as shedding *E. coli* O157:H7. Unfortunately, the exact number of *E. coli* O157:H7 was impossible to determine due to the presence of sorbitol fermenting colonies.

Group 2. Eight weaned Holstein-cross calves reared conventionally on a farm and aged between 7 to 12 weeks were used. Calves were housed together and fed concentrate twice daily and had access to *ad lib* hay and water. Animals were challenged four times with $\times 10^{9}$ CFU (day 0), 1×10^{10} CFU (day 7) and 2×10^{10} CFU of *E. coli* O157:H7 on days 35 and 36.

Group 3. Four weaned Holstein-cross calves reared conventionally on a farm and aged between 8 to 14 weeks were orally challenged via stomach tube on a single occasion with $\times 10^{9}$ CFU for ultrastructural investigations.

Group 4. Due to the shortage of fresh mucosal tissues at the end of the PhD period, additional samples for antibody detection assays were kindly donated by Dr. T. McNeilly from a separate experiment. Six weaned Holstein-cross calves reared conventionally on a farm and aged between 7 to 11 weeks were orally challenged via stomach tube with $\times 10^{10}$ CFU of *E.coli* O157:H7. Rectal swabs, nasal secretions and serum samples were taken 4 days pre and 2 weeks post-challenge. These samples had confirmed IgA and IgG1 serum and rectal mucosal antibody responses

against O157 and H7 antigens measured by Enzyme –linked immunosorbent assay (ELISA) methods and results have been described elswhere (McNeilly et al., 2007).

Group 5. Five, unexposed, culture negative calves of similar age, breed and background were used as controls for the histopathological and immunological studies.

2.3 Bacterial detection in faeces, gastrointestinal contents and *post-mortem* tissues

Faecal samples were collected upon defecation from the animal following stimulation of the anal sphincter, the faecal surface was sampled with wooden spatulas and then the core exposed for sampling. Shedding was regularly monitored by faecal culture. *E. coli* O157:H7 per gram of faeces or per square centimetre of *post-mortem* tissue was enumerated using the following procedures: Ten grams of faeces or gastrointestinal contents were serially diluted in PBS using 10 fold steps. Squares of gastrointestinal tissue samples were put into 5 ml of PBS, vortexed for 60 s, and the supernatants serially diluted ten fold in PBS. 100 µl aliquots of the serial dilutions were cultured onto sorbitol MacConkey agar (SMAC, Oxoid) containing 15 µg ml^{-1} of nalidixic acid (Sigma-Aldrich) in triplicate for faecal samples or duplicate for tissue washings. After overnight incubation at 37°C non-sorbitol fermenting colonies were counted and one colony from each sample tested using a latex agglutination test kit (Oxoid) for O157 lipopolysaccharide. Plates with less than one hundred colonies were counted manually, an automated system (Acolyte) was

used to enumerate plates with over a hundred colonies. The minimum detection level was 33 colonies per g^{-1} of faeces.

2.4 Rectal biopsy

Rectal biopsies were taken under local anaesthesia achieved by epidural administration of 1/2 ml of Lidocaine (Lignol, Arnolds,) into the intercoccygeal space between C1-C2. The biopsies were excised from the terminal rectum by using forceps and scissors, to minimise animal discomfort specimen size consisted of small pieces of rectal mucosa weighing between 50 and 75 mg (3/4 mm^{2}). The tissues were placed in self sealed plastic bags and were immediately frozen by immersion of the sample in liquid nitrogen and stored at -70 °C.

2.5 Intestinal mucosa, serum and saliva collection

The following samples were collected from the groups described previously in section 2.2.

Group 1: A total of 22 animals from group 1, still shedding two weeks post-initial challenge, were examined *post-mortem* and various tissue and contents samples (described in section 2.7) collected from the gastrointestinal tract for bacterial counts. Rectal mucosa (weighing approximately 2 g) was excised at post-mortem examination from 9 animals, frozen by inmersion of the sample in liquid nitrogen and stored at -70°C. Rectal biopsies were also taken from 11 of these animals pre-challenge and on two further occasions at 6 and 12 days after challenge and processed for histological examination (Annex Table V). Six animals

were challenged rectally and five orally. Serum samples were collected pre-challenged and at *post-mortem*.

Group 2: A total of 32 rectal mucosal biopsy samples were collected at 28 days pre-challenge and 6, 34 days post-challenge and during post-mortem examination at day 54. On the first three occasions, tissue was excised under local epidural anaesthesia, i.e. before initial challenge and on two further occasions at 6 and 34 days after challenge. The last samples were taken during post-mortem examination.

Group 3: Sections of rectal mucosa were excised *post-mortem* and tissues fixed and processed for electron microscopy and immunogold labelling as described in section 2.12.

Group 4: Nasal secretions were collected by placing one regular size non-applicator tampon (Lillets, Accantia, Birmingham, UK) into the nostril of each calf for approximately 10 mins. Nasal secretions were subsequently expelled by squeezing within the barrel of a 20ml syringe. Rectal swabs were collected by gently swabbing the last 3/4 centimetres of the rectal mucosa. The cotton swab was then placed in 1m of PBS, and spun to remove the gross particulate portion. The supernatant was collected and stored at -20°C. Serum samples were collected 28 pre-challenge, 14, 35 and 49 days post-challenge.

Group: 5 Sections of rectal mucosa were excised *post-mortem* and two sets of tissues taken for histology or freezing at -70°C in liquid nitrogen.

2.6 Generation of rectal mucosal extracts

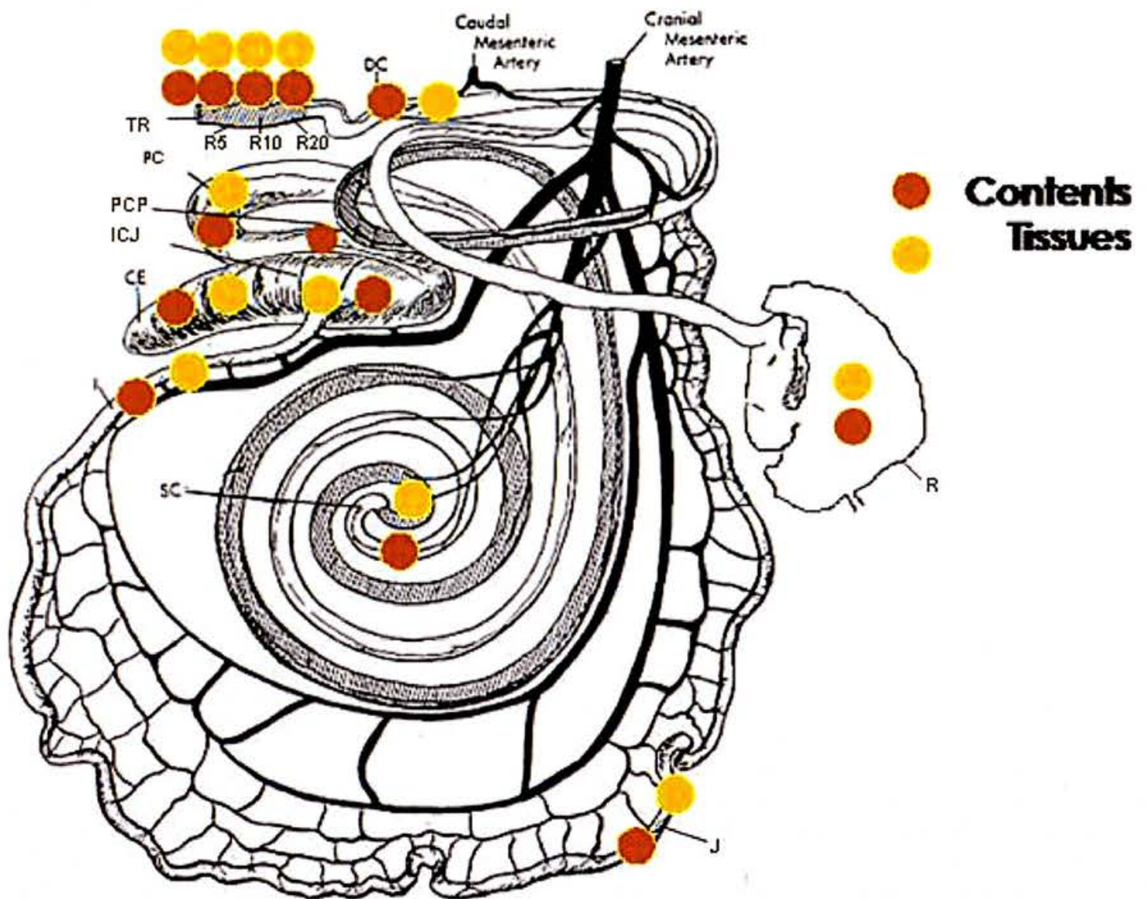
After thawing, 50-75 mg of the biopsy or rectal mucosal tissue removed at *post-mortem* was mixed with 1 ml of ice cold phosphate buffered saline (PBS, pH 7.2) and protease inhibitor cocktail (Roche) in RiboLyzer™ tubes (Hybaid, United Kingdom), and then processed in a Hybaid RiboLyzer™ for 10 s at 5.5 ms^{-1}. The supernatant of the resultant extract was twice removed and centrifuged at 2,000 x g to remove the tissue remnants. The protein content was standardised to a final concentration of 5mg/ml for group 1 and 3 mg/ml for group 2.

2.7 Necroscopy

A sample of naturally voided faeces was collected prior to euthanasia of these animals with intravenous pentobarbitone (140mg/kg, J.M.Loveridge Ltd). Once death was confirmed, a blood sample was taken by incising the jugular vein while the animal was hanging from a hoist. First, a circular cut was made around the anus, taking care to leave the anal sphincter intact. The subsequent cuts free the anus, rectum and bladder from surrounding tissue. Then, the skin and abdominal muscles were incised along the ventral midline, and the pelvic cavity was accessed from the abdominal cavity by cutting the peritoneum. The rectum was sectioned between two ligatures and separated from the rest of the gastrointestinal tract, the urinary bladder was removed. The rest of the gastrointestinal tract, from the distal colon to the oesophagus was removed from the carcass and placed over a table. The contents were then sampled from: rumen, gallbladder, jejunum, ileum, caecum, proximal colon, spiral colon, distal colon, and at four different levels of

the rectum (20, 10 and 5 centimetres before the pectinate line that delimits the end of the rectal mucosa). Two sets of tissues for histology and for bacterial counts were taken at: rumen, jejunum, ileum, caecum, ilocaecal valve, proximal colon, proximal colon patch, spiral colon, distal colon and at proximal, mid, distal and terminal rectum (20, 10, 5 and 1 centimetres from the pectinate line, Figure 2-1).

Figure 2-1. Sampling sites for collection of contents and tissues



R	Rumen	SC	Spiral colon
J	Jejunum	DC	Distal colon
I	Ileum	R20	20 cm from terminal rectum
ICJ	Ileo caecal junction	R10	10 cm form terminal rectum
PCP	Proximal colon patch	R5	5 cm from terminal rectum
PC	Proximal colon	TR	Terminal rectum

2.8 Fixatives, solutions and media

The list of tissue fixatives, solutions and media used is presented in Tables 2-2, 2-3 and 2-4. All solutions were made up in dH2O and sterilised by autoclaving at 15 pounds per square inch for 20 mins prior to use.

Table 2-2. Tissue fixatives

Fixative	Composition
4% w/v Paraformaldehyde	900 ml of PBS were pre-heated at 60° in a Duran bottle and 40g of paraformaldehyde (PFA) added inside a fume cupboard. The mix was left on the heating/stirring block until total solution, and stored at 4° for up to15 days.
Zinc Salts Fixative	Tris-Calcium acetate buffer (Trizma base 12.1g calcium acetate 0.5g, distilled water up to 1,000ml) was made and pH adjusted to 7 – 7.4, followed by the addition of 5g each of Zinc chloride and Zinc acetate. The fixative can be stored at room temperature for up to six months.

Table 2-3. Solutions

Solution	Composition
Phosphate Buffered Saline	137mM NaCl, 10mM Na2HPO4 2.7mM KCl, 1.4mM K2HPO4, adjusted to pH 7.4 (Oxoid)
Tris-Borate-EDTA	(TBE) 108 g Tris base. 55 g Boric acid. 9.3 g Na_4EDTA. Add dH2O to 1 liter. pH 8.3
PBS/NaCl/T80 buffer	PBS, 0.5% Tween® 80 and 0.5 M NaCl
10x TBE	1 litre contained 0.9 M Tris base, 0.9 M boric acid and 0.02 M EDTA (disodium salt) dissolved in dH2O
10x loading buffer	For nucleic acid gel electrophoresis contained 15% (w/v) Ficoll with 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene green.
Ethidium bromide	Stock solution of 10 mg ml^{-1}, dissolved in dH2O and stored at room temperature in the dark.
Scott's tap water	Magnesium sulfate, 200 g/l, sodium bicarbonate, 20 g/l, and preservative.

Lysis buffer	10 mM Tris-HCL (ph 7.5), 0.5 mM PMSF, aprotinin 0.5 µg/ml
Sarkosyl buffer	100 mM NaCl, 10nM Tris-HCl ph 8.0, 0.5 mM PMSF, aprotinin 0.5 µg, 0.5% N-lauroylsarcosine (Sigma)
TBF1	30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % (v/v) glycerol, adjusted to pH 5.8 with acetic acid
TBF2	10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15 % (v/v) glycerol, adjusted to pH 8.0 with KOH

Table 2-4. Media

Media	Composition
Luria Bertani broth (LB)	Contained Difco Bacto tryptone (10 g), Difco Bacto yeast extract (5 g) and NaCl (10 g) dissolved in 1 l dH2O. The pH was adjusted to 7.2 with 5 N NaOH and the media autoclaved (20 lb/in^2, 120°C for 15 mins).
LB Agar	LB broth was converted to solid media by adding Difco agar (15 g l-1) prior to autoclaving.
SOC	The medium contained Difco bacto-tryptone (20 g), Difco yeast extract (5 g), NaCl (10 mM), KCl (2.5 mM), MgCl2 (10 mM), MgSO4 (10 mM) and glucose (20 mM) dissolved in 1 litre of dH2O.
LBC	LB Agar containing 25 µg/ml of Chloramphenicol
LBK	LB Agar containing 12 µg/ml of Kanamyacin
LBA	LB Agar containing 12 µg/ml of Ampicillin

2.9 Histopathological studies

Tissues taken at post-mortem examination for histopathology were immediately fixed in 4% paraformaldehyde or Zinc salts and sections staining performed on 5 µm sections on labelled glass slides. Sections were first hydrated in descending xylene and alcohol solutions (100%, 90%, 80%, 70%, and 50%), stained with the appropriate technique and rehydrated in ascending alcohol solutions clear with xylene and a coverslip mounted. Histochemistry was performed manually following the

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protocols described below and a coverslip was mounted in a solvent-based medium (Table 2-5). Due to logistical constraints, histological examination was limited to the first thirteen of twenty two calves sampled for tissue counts from group 1.

Table 2-5. Histochemical stains

Staining	Procedure
Hematoxylin-and-eosin	1. Immerse sections in Harris Hematoxylin (Cell Path) for 1 min. 2. Rinse with water. 3. Exchange tap water until the water is clear. 4. Immerse sections in Eosin stain for 1-2 mins. 5. Rinse with water. 6. Exchange tap water until the water is clear.
Toluidine Blue	1. Stain in 0.5% Toluidine Blue in HCl pH 0.3 for 1 hour (Philip Harris). 2. Wash briefly in water. Results: Mast cells appeared deep purple. The background is pale blue colour with nearly no nuclear staining.
Carbol Chromotrope	1. Nuclei were stained with haematoxylin for 1 min. 2. Rinse with water. 3. Stained for 1 hour in Carbol chromotrope solution (Sigma), 0.5% chromotrope 2R in 1% phenol. 4. Rinse with water. 5. Results eosinophil granules, bright red. Nuclei blue.

2.10 Immunohistochemistry

For immunoperoxidase staining deparaffinised and rehydrated sections which were mounted onto Sequenza slide racks (Thermo Electron, formerly Shandon) and treated as follows: To block endogenous peroxidases, 3% H_2O_2 was added to each section and sections were incubated at room temperature for 5 mins. To block non-specific binding,

a 25% solution in TBS of goat serum was added for 30 mins. Primary antibody was then added at a 1:1,000 dilution and incubated at 4°C overnight. The primary antibody was detected using EnVision™/HRP Detection System Peroxidase/DAB (Dako) used accordingly to manufacturers instructions. Briefly, secondary peroxidase-conjugated anti-rabbit goat raised antibody diluted to 1:1,000 was added for 30 mins, followed by 3,3'-diaminobenzidine (DAB) substrate in chromogen and incubated for 7.5 mins. Finally, slides were counterstained with Haematoxylin (CellPath) for 30 s, rinsed in distilled water and blueing achieved by immersion in Scott's tap water substitute solution (Sigma-Aldrich) for 1 mins followed by a rinse with distilled water. Sections were dehydrated in ascending alcohol solutions and a cover slip added over a solvent-based mountant solution. The total amount of reagent used per slide was 100 µl. Working dilutions of all reagents were made in TBS. On each stain run two types of controls were used . As a non-reagent negative control, one section in each run was incubated with PBS lacking the primary antibody.

For immunofluorescence microscopy sections were first dewaxed in xylene and rehydrated with serially graded ethanol dilutions in water. The sections were incubated in Sequenza racks with rabbit anti-O157 polyclonal antibody (Mast-Assure) at 1 in 100 dilution of PBS for 30 mins at room temperature. Sections were then trated with fluorescein isothiocyanate (FITC) conjugated goat antirabbit antibody (Mast-Assure) at a 1 in 1,000 dilution in PBS for 30 mins. Cover slips were added after application of Fluorescence Mounting Media (Dako). For confocal

microscopy tissue was examined with a Zeiss 510 confocal microscope with a 63× objective lens.

In each immunostaining run negative/positive control slides were included. As a non-reagent negative control, one section was incubated with PBS lacking the primary antibody. A positive control specimen using a tissue known to contain the antigen was also included in each assay for *E. coli* O157:H7 and a positive internal control using lymphoid follicles as normal elements that express the antigen of interest, was used to asses MHC class II expression.

2.11 Antibodies

The antibodies used for immunohistochemistry, immunoblotting and ELISA are listed in Table 2-6.

Table 2-6. Antibodies

Antibody	Manufacturer	Working concentration
Polyclonal sheep anti-bovine IgA HRP	Serotec	1:1,000
Polyclonal sheep anti-bovine IgG1 HRP	Serotec	1:1,000
Polyclonal rabbit anti-*E. coli* O157 LPS	Mast Assure	1/100
Monoclonal mouse anti-TCR IL A29	International Livestock Research Institute	1/1,000
Monoclonal mouse anti- β-chain MHC II SW.73.2	Moredun Research Institute (Hopkins et al., 1986)	1/10,000
Monoclonal mouse anti-H7 flagellin	Mast Assure	1/1,000
Goat anti-rabbit IgG 10nm gold labelled antibody	British Biocell	1/500
Mouse anti-bovine IgA	Dako	1/500

Biotinylated goat anti-mouse immunoglobulin	Dako	1/2,500
Streptavidin-horseradish peroxidase	Dako	1/4,000
FITC-conjugated goat anti-rabbit	Mast Assure	1/1,000

2.12 Electron Microscopy

Two different techniques were used to identify *E. coli* O157:H7: (i) conventional transmission electron microscopy (TEM) on reprocessed tissues from paraffin blocks and (ii) gold labelling and TEM on tissues specifically fixed for immunolabelling, in order to improve the quality of the samples. For conventional TEM, areas of three tissue sections from two animals from group 1 with the highest density of *E. coli* O157:H7 attached (identified by IFA) were circumscribed and the tissue in the matching areas of the paraffin blocks excised precisely and reprocessed for TEM as described elsewhere (Wales et al., 2001a). In brief, three such regions from two animals were then deparaffinised with xylene, rehydrated 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. Seven ultrathin sections (80 nm) were cut from each of the three regions identified above and mounted on copper grids (Agar Scientific). Random regions negative for *E. coli* O157:H7 microcolonies were similarly processed, and no AE lesions were detectable by TEM

Immunogold labelling was carried out in samples from the only two successfully colonised animals out of four challenged specifically for

electron microscopy examination from group 3. Terminal rectum tissues were fixed in 2% formaldehyde and 0.1% glutaraldehyde and labelling of bacteria was made with the same primary antibody used for light microscopy, with a secondary goat anti-rabbit IgG 10um gold labelled antibody (British Biocell, 1:500 for 60 mins at room temperature). As a non-reagent negative control, one section was incubated with PBS lacking the primary antibody. Specimens were viewed on a Philips CM12 transmission electron microscope.

For scanning electron microscopy (SEM), all specimens were placed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.3) overnight and then immersed in 2% guanidine hydrochloride and 2% tannic acid for a second night. They were subsequently post fixed in 2% osmium tetroxide in distilled water for 8 hours. Dehydration in graded concentrations of acetone (50%, 70%, 90%, 100%) was followed by critical point drying using carbon dioxide. After they were mounted on aluminium stubs, the specimens were sputter coated with 20 nm gold/palladium and viewed with a Hitachi S4700II field emission scanning electron microscope using appropriate settings. Digital images were recorded using Hitachi FE PCSEM 3.2. Images were processed with PaintShop Pro (Jasc Software).

2.13 Tissue and bacterial counts

Cell counts were made of total granulocytes, eosinophils, mast cells and γδ T cells in the lamina propria of the rectum. Total granulocyte numbers were counted in sections stained with hematoxylin and eosin, mast cells

from sections stained with toluidine blue (0.5% in 0.5N HCl, pH 0.5) for 1 hour (Enerback, 1966) and eosinophils in sections stained with carbol chromotrope solution for 1 hour and counterstained for 10 s with hematoxylin (Lendrum, 1944). IL A29 was used to detect the gamma/delta (γδ) T cell marker BoWC1 (Howard et al., 1991) in immunostained sections. Considering a power of 0.8 and 95% significance level, counts were made on five fields on sections from two different paraffin blocks. Counts were expressed as cells per 0.25 mm^2.

2.14 Statistical analysis

E. coli O157:H7 counts within faecal and tissue samples were calculated by determining the mean plate count at the most relevant dilution for each sample, and by multiplying the dilution factor to convert to CFU g^{-1} (faeces or GIT contents) or CFU cm^{-2} (mucosal samples). The concentration (CFU g^{-1} plus 1) was log_{10} transformed. Student's T-test was used to compare means of samples, with a paired T-test being used where a natural pair existed. The chi-square test was used to analyse proportions. Wherc the numbers of observations were low in some categories then Fisher's exact test was used. The Kappa test was used to qualify the level of agreement, where the null hypothesis is that there is no more agreement than might occur by random chance. Data was processed using Excel (Microsoft) and Minitab (Minitab Inc) software. The applicability and suitability of the tests were discussed with Dr. Iain McKendrick and Dr. Jill Sales (Biomathematics and Statistics, Scotland Research Institution).

2.15 Antigens

Whole cell samples of *E. coli* O157:H7 ZAP 198, *E. coli* O26 ZAP 194 and *E. coli* K12 ZAP 185 were prepared from overnight LB broth cultures, followed by 2 consecutive 4,000 x g for 5 min. and resuspension in PBS, and sonicated on ice twice for 10 s at 15 microns with 10 s pauses (Soniprep 150, MSE).

Bacterial membranes and cytoplasmic proteins preparations of *E. coli* O157:H7 ZAP 198 were obtained by harvesting bacteria in mid-log phase after overnight growth in LB broth. Bacteria was pelleted by centrifugation (13,000 x g for 5 min) and resuspended in 8ml of lysis buffer, freeze-thawed four times in dry ice, and sonicated on ice twice for 10 s at 15 microns with 10 s pauses (Soniprep 150, MSE). Whole bacteria and cell envelopes were removed by centrifugation (2 x 5,000 for 10 min at 4^{0}C). The supernatant containing the cytosolic soluble proteins and the insoluble inner and outer membrane proteins was removed, and ultracentrifugated for 1 hour at 50,000 x g at 40C to pellet the membranes. The resultant supernatant containing the cytoplasmic proteins was collected and concentrated to 0.5 ml with 100 Kda molecular weight cut-off centrifugal filter devices (Millipore Corporation). The membrane pellet was washed twice and resuspended in 0.5 ml of lysis buffer.

Lysates of *E. coli* O157:H7 ZAP 198 were made by two methods: Trypsinisation of whole cell samples for 45 mins at 70°C with Sequencing Grade Modified Trypsin (Promega). Proteinase K treated lysates were

prepared by diluting 5 µg of crude bacterial protein into 8 µl of SDS-PAGE lysis buffer and 3 µl of reducing agent per gel lane. The preparation was incubated with 176 µl of proteinase K (25 mg/ml, specific activity: ≥ 30U/mg) (Roche) at 60° C for 1 hour.

Supernatants containing **Type III secretion system proteins** were produce by growing bacteria in minimal essential medium modified with 25 mM HEPES (Sigma-Aldrich) to an optical density of 600 nm. Suspensions were centrifuged at 4,000 x g for 10 min. The supernatant was filtered and the proteins were precipitated by the addition of 10% trichloroacetic acid with overnight incubation at 4 °C, followed by centrifugation at 4,000 x g for 30 min. The protein pellet was suspended in 1.5 M Tris (pH 8.8) and after addition of sample buffer, the proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining as previously published (Kenny and Finlay, 1995).

***E. coli* O157:H7 LEE4 mutants** were used as negative controls and obtained from stocks of previously published work (Naylor et al., 2005b).

Flagellin was purified by dissociation of the flagella from bacteria by adjusting the pH of the bacterial suspension to 2.0 with 1 N hydrochloric acid for 30 min. Bacteria devoid of flagella were harvested by centrifugation at 5,000 x g for 30 min. The supernatant containing flagellin was centrifuged at 100,000 x g for 1 h at 4°C, and the supernatant was adjusted to pH 7.2 with 1 N sodium hydroxide. Flagellin was precipitated by incubation with 2.67 M ammonium sulfate for 16 h

at 4°C and collected by centrifugation at 15,000 x g for 15 min. The precipitate was re-suspended in distilled water and dialysed in tubing with a 50,000-molecular-weight cutoff against running water at 4°C for 24 h (Sherman et al., 1988). This protocol results in the purification of flagellin monomers, which spontaneously repolymerise into flagellar filaments at neutral pH (McNeilly et al., 2008).

O157 Lipopolysaccharide (LPS) was obtained from commercial sources (List Biological Laboratories).

Outer membrane porin C (OmpC) external loops 1, 2, 4, 5 and 6 were solid-phase synthesised to a purity of >70% (Thermo Electron Corporation). The sections were between 12 to 17 amino acids long, and contained the external hairpin connecting the anti parallel beta strands that form the main barrel of the porin. Peptides (1mg/ml) were dissolved in PBS and stored at -20°C.

Cytoplasmic preparations were kindly supplied by D. Wang and secreted proteins and flagellin preparations were kindly donated by S.P. McAteer (Zoonotic and Animal Pathogens Laboratory, University of Edinburgh)

2.16 Protein determination

To determine sample protein concentration a Pierce Coomassie Protein Assay Kit (Pierce Biotechnology) was used according to the manufacturer's instructions. The colorimetric absorbance was read at a wavelength of 562 nm in a Dynex Revelation 4.02 (Dynex Technologies) Spectrophotometer.

2.17 ELISA

E. coli O157 LPS and H7 flagellin preparations were used to coat ELISA plates to determine mucosal and serological IgG1 and IgA antibody titers. To coat ELISA plates (Thermoelectron 3455) O157 LPS (List Biological Laboratories) was conjugated to polymyxin B as previously described (Currie and Poxton, 1999). Wells were coated overnight at 4°C with 1 µg of H7 and O157 LPS in 0.05 M Carbonate buffer, pH 9.6. After washing two times with PBS pH 7.4 containing 0.05% Tween® 20 (PBS/T20), blocking was performed with 3 % fish gelatin (Sigma G7765) followed by sequential incubation with dilutions of primary antibody for 1 hour at 37°C in PBS/NaCl/T80. The primary antibody was used at a concentration of 0.5 mg/ml of total protein for rectal extracts and to a dilution of 1/5 for sera IgA and 1/100 for sera IgG1. The secondary sheep anti bovine IgA or IgG1 HRP conjugated (Serotec) antibody was then added (1/1,000 PBS/NaCl/T80) for 1 hour at 37°C, and Sigma Fast OPD added until colour developed. Between each incubation, plates were washed six times with PBS/T. Finally, the reaction was stopped with 25 µl of 3M sulphuric acid (Fisher S/9160/PB15) and the O.D. readings obtained at 492 nm. For each plate, four wells were incubated with PBS/NaCl/T80 alone (negative control). Each sample was analysed in duplicate and final absorbance calculated by subtracting the average absorbance of the negative controls. A pool of rectal extracts from three positive animals from Group 1 with detectable histological lesions and previously found to be positive by immunoblotting, were used as a positive controls. A similar system was used to detect OmpC external loop peptides (1 µg per well in 0.05M Carbonate buffer) were used to coat

the plates to detect the presence of mucosal and serological IgG1 and IgA antibody responses. Primary antibody was used at the following dilutions: Nasal secretions 1:10, rectal swabs 1:10 and serum at 1:100 dilutions. The ELISA had been previously developed by A. Flockhart.

2.18 Dot blots

10 μg of each purified peptide were applied directly to a nitrocellulose membrane (Invitrogen) as spots in eight serial dilutions from 1:1 to 1:32. The membranes were incubated at room temperature with nasal secretions (1:10), rectal swabs (1:10) and serum (1:100) diluted with PBS to achieve the required concentrations. The membranes were rinsed in PBS/NaCl/T80 and incubated with secondary anti bovine IgA or IgG1 HRP conjugated (Serotec) antibody at a dilution of 1:500 for 1 hour. The membrane was washed again and the signal was detected using the ECL system (Amersham Bioscience, Piscataway, NJ). The membranes were exposed to the film for 15 s (Super RX, Fuji). Whole cell preparations of *E. coli* O157:H7 were used as a positive control, and PapB from *E. coli* CFTO73, a fimbrial regulator present only in uropathogenic isolates, was used as a negative control.

2.19 One dimensional SDS-PAGE

For one dimensional SDS-PAGE (1-D SDS-PAGE), the bacterial antigen was heated for 10 min at 70°C in loading buffer, 5 μl of SDS and 2 μl of reducing agent (Invitrogen). Each lane was loaded with 5 μg of bacterial protein and separated using 4%-12% NuPAGE Novex bis-tris gels (Novex,

Invitrogen, USA) with NuPAGE MES SDS running buffer. Proteins in the gel were visualised using Simply Blue stain (Novex, Invitrogen).

2.20 Two-dimensional gel electrophoresis

Two-dimensional (2D SDS/PAGE) separation of proteins was carried out by isoelectric focusing using the ZOOM IPGRunner System followed by separation by protein size on Bis-Tris Zoom gels SDS/PAGE utilizing the NuPAGE Novex 4-12% Bis-Tris ZOOOM gels (Invitrogen) according to the manufacturer's instructions. Interfering charged molecules and DNA were removed using a 2-D Clean-Up kit (Ettan kit, Amersham) according to the manufacturer's instructions. Sample loads were used as recommended by the manufacturer (50 μg for Western blotting and 200 μg for staining). Samples were subsequently suspended in 155 μl of buffer containing 20 mM DTT, 8M urea, 2% CHAPS and 2% ZOOM carrier Ampholytes pH 3.5-10 (Invitrogen). IEF ph 3-10 strips were rehydrated overnight with the sample buffer and isoelectric focusing performed for 4 h at 500 V. Following protein separation by charge, the IEF strips were incubated for 15 mins in reducing buffer (NuPAGE, Invitrogen) followed by 15 mins in an alkylating solution at room temperature. The resultant 2-DE SDS-PAGE gel was stained for protein by Simply Blue stain (Novex, Invitrogen). Following the manufacturers suggestions two sample loads were used, 100 μg of crude bacterial protein for Immunoblotting and 20 μg for protein staining.

2.21 Western blotting

Gels were transferred onto nitrocellulose membranes (Invitrogen) using a semi-dry transfer apparatus at room temperature. After transfer the membranes were incubated in PBS/NaCl/T80 buffer for 60 mins at room temperature to block non-specific protein binding. The transferred proteins were incubated with the supernatant from the mucosal extracts (0.03 mg/ml of mucosal protein). Following three washes, the membranes were incubated sequentially with mouse anti-bovine IgA (Dako, USA, 1/500 in PBS/NaCl/T80), biotinylated goat anti-mouse immunoglobulin (Dako 1/2500 in PBS/NaCl/T80) and streptavidin-horseradish peroxidase (Dako, Streptavidin-HRP, 1/4000 in PBS/NaCl/T80). The incubation steps were performed for 60 mins at room temperature with three washes with PBS/NaCl/T80 between each step. Peroxidase activity was revealed by chemiluminescence using ECL reagent (Amersham Life Sciences, Bucks, UK). Control Western-blots consisted of incubations with bovine rectal extract at the same concentration from: a) pool of 3 animals with histopathological lesions (positive controls): b) from unexposed animals (negative control); c) Western blots in which no primary antibody was added.

2.22 Protein molecular weight marker

To determine the size of the proteins a SeeBlue Plus2 Pre-Stained Standard (Invitrogen) marker with a molecular weight range of 4–250 kD was used.

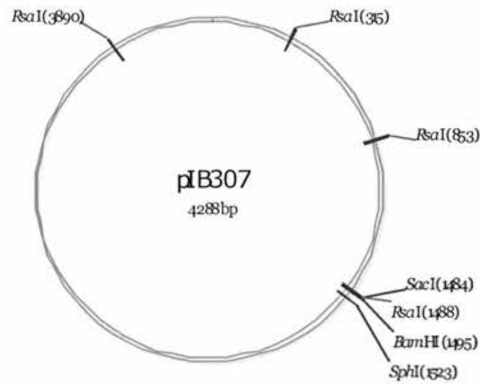
2.23 MALDI-ToF/TOF mass spectrometry

Immunoreactive spots were overlapped to 2-D SDS/PAGE gels and the matching protein identified and excised from 2-DE gels, destained and digested overnight with 20µl of porcine trypsin solution at 1µg/µl (Trypsin Gold, Promega) resuspended in 50mM acetic acid and then diluted in 40mM NH4HCO3/10% ACN to 20µg/ml. Resultant digests were analysed on a matrix-assisted laser desorption ionisation in tandem time-of-flight spectrometry (MALDI-TOF/TOF) (Broker Ultraflex II biospectrometry workstation, Applied Biosystems). The fragmentation data obtained were handled using the MASCOT software package and a Mowse score was calculated according with scores greater than 74 indicative of significant protein identity.

2.24 Plasmid and plasmid constructs

The main reagent in the gene replacement is the plasmid pIB307 (Figure 2-2). This replicon is unable to replicate over 28°C. The plasmid restriction sites used in this study were *Sph*I at 1523 bp, *SacI* at 1,484 bp, *Bam*HI at 1,495 bp, and three more sites for *Rsa*1 at 315, 853 and 3890 bp.

Figure 2-2. Plasmid pIB307 size and homologous regions insertion sites



The plasmids used in this work are listed in Table 2-7.

Table 2-7. Plasmids

Plasmids	Description	Source/Reference
pIB307	4,288 bp. Low copy (6-8 copies/bacterium). Chloramphenicol resistance cassette. Temperature sensitive replicon, unable to replicate over 28°C.	(Blomfield et al., 1991)
pPN1	5,288 bp. Formed after insertion into the pIB307 backbone of the 1,000 bp right flanking region of *ompC,* amplified using primers ompCRHF1 and ompCRHF2 (Table 2-10).	This thesis
pPN2	6.421 bp. Formed after insertion into the pIB307-PN1 backbone of the 1,133 bp left flanking region of *ompC* amplified using primers ompCLHF1(f) and ompCLHF2 (Table 2-10).	This thesis
pPN3	10,251 bp. Formed after the insertion of the 3,830 bp *sacB-Kan* cassette between both *ompC,* homologous flanking regions of PN2.	This thesis
pKC11	pIB307 with CFT073 *lacI* and *lacA* regions, and *sacB-Kan* inserted between *lacIA*	(Holden et al., 2006)
pCR4-TOPO	3,900 bp. Directional TOPO® Cloning vectors. Confers Ampicillin resistance.	(Shuman, 1992)

	*Eco*RI sites flank the PCR product insertion site.	
pET100–R	4,900 bp. Formed after insertion of the right flank into the pIB307 backbone	This thesis
pET100–L	5,033 bp. Formed after insertion of the left flank into the pIB307 backbone	This thesis

2.25 Plasmid purification

Plasmid DNA was purified from lysed bacterial pellets obtained from 1.5 ml of overnight cultures, using the Plasmid Mini-Prep Purification Kit (Qiagen) following manufacturer's instructions.

2.26 Restriction digests

Restriction endonucleases were purchased from New England Biolabs and used according to the manufacturer instructions (Table 2-8). 1 µl of the enzyme was added to 50 µl volume containing dH_2O, bovine serum albumin (100 µg/ml) and different proportions of DNA suspension. Digests were incubated at 37°C for the manufacturer's specified time. DNA was purified using Qiaprep Spin DNA Miniprep purification kit (Qiagen) according to the manufacturer's protocol and reconstituted in 30 µl of dH_2O.

Table 2-8. Restriction endonucleases

Enzyme	NEB buffer	Incubation temp. (°C)	Incubation time	Recognition Site (5' to 3')	Recognition Site (3' to 5')
*Bam*HI	3	37	2-12 hours	G▼GATCC	CCTAG▼G
*Sp*HI	2	37	4-12 hours	GCATG▼C	C▼GTACG
*Sac*I	1	37	2-12 hours	GAGCT▼C	C▼TCGAG
*Eco*RI	1	37	1 hour	G▼AATTC	CTTAA▼G
*Rsa*I	1	37	1 hour	GT▼AC	CA▼TG

To confirm that the plasmids have the desired construct a series of diagnostic digests were performed as described in table 2-9.

Table 2-9. Diagnostic digests

4 µl	Plasmid preparation
1 µl	Enzyme
2 µl	10x Buffer
2 µl	10x BSA
11 µl	RNA/DNAse free water

2.27 Primers

Primers used in the study are listed in Table 2-10. The restriction enzyme cutting sites are underlined.

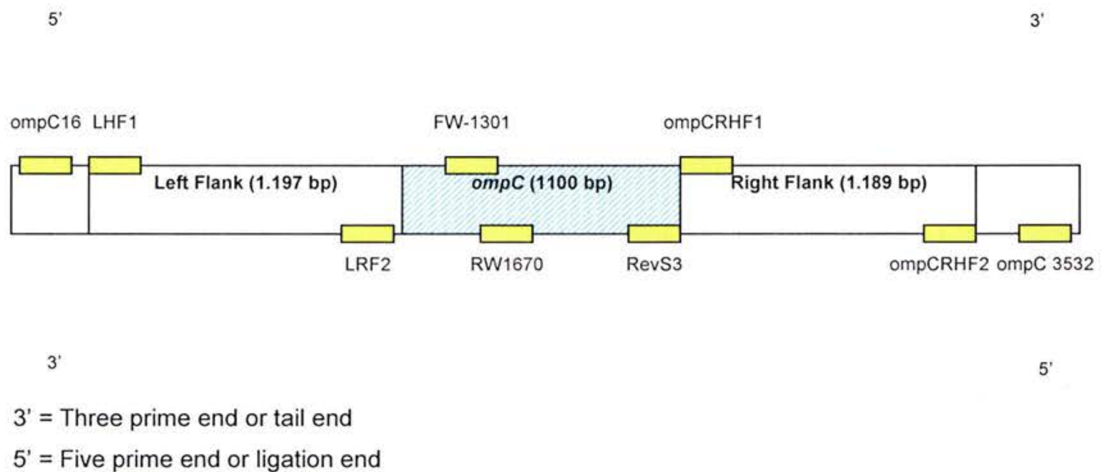
Table 2-10. Primers

Primer No.	Sequence 5' 3'	Paired primer	Annealing Temperature	Product size (bp)
	***ompC* flanking regions amplification**			
ompCRH F1	CGGGATCCTCTCGATTGATATCGAACAA AGG			
ompCRH F2	AATTGCATGCCGGTGCAATCACCGTCAC G	ompCRHF1	60.0 °C	1,000
ompCLH F1(f)	ACGTGAGCTCATCCGTTGAGTCATCTCA AGC			
ompCLH F2	GCGGATCCTTAACCCTCTGTTATATGCCT	ompCLHF1(f)	56.0 °C	1,133
	***sacB-Kan* insertion verification**			
pIB307 F	AGACAAATGGATCTCGTAAGCG			
pIB307 R	GCTGTAACAAGTTGTCTCAGGTGT	pIB307 F	52.7 °C	500
sacB-Kan F	CAGCTCTTTGAACATCAACGG			
sacB-Kan R	CTTGGTAGCCATCTTCAGTTCC	sacB-Kan F	54.8 °C	200
ompC16	GGTAATACATTGACCACG			
RevS3	CCCAAGCTTTTAGAACTGGTAAACCAGA CCCAGAGCCCAGAGCTACGATGTT	ompC16	59.1 °C	2,302
ompCRH F1	TCTCGATTGATATCGAACAAAGG			
ompC 3532	TCCCTGGTTAAGGATAGC	ompCRHF1	54.8 °C	1,232
	SAC-KAN insertion			
FW 1131	GAGGGTTAATCAGTATGCAG			
RW 2390	ACGGTCGCAAGAGTACACCA	FW 1131	54.0 °C	3,989
	Detection of *ompC* deletion			
FW 1301	GGTAAAGTAGACGGCCTGC			
RW 1670	CGAAGAAGTCAGTGTTACGG	FW 1301	54.8 °C	370

2.28 Schematic representation of gene *ompC* and primer positions

An arbitrary bacterial DNA nucleotide upstream of the left flanking region (for a detailed sequence see Table 14) was numbered as base number one. The primer's name and position on the bacterial genome is specified for each primer in Figure 2-3.

Figure 2-3. Starting point of primers replication on *ompC* gene and flanking regions

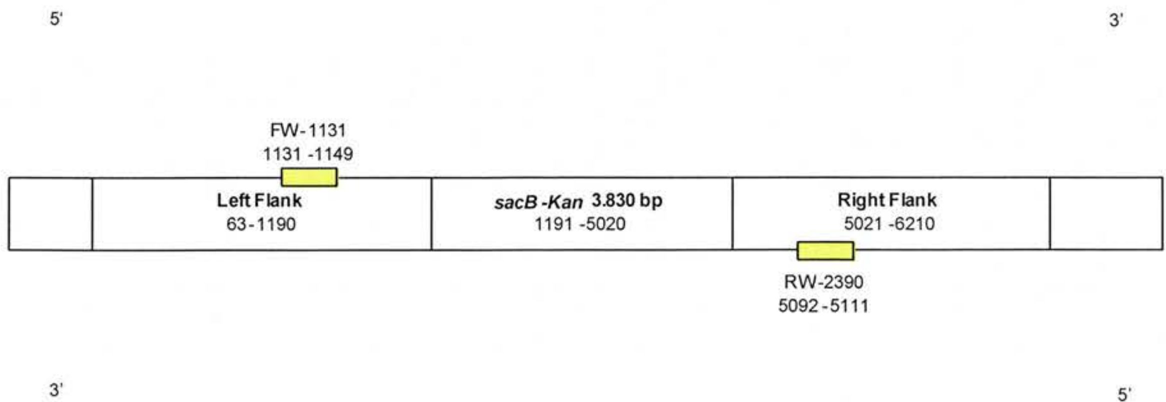


3' = Three prime end or tail end
5' = Five prime end or ligation end

2.29 Schematic representation of *sacB-Kan* insertion and primer positions

The same first nucleotide is used for enumeration purposes in the following diagram of the final construct with the exchanged region inserted in place of the *ompC* gene (Figure 2-4). Insertion of the larger (3,830 bp) *sacB-Kan* cassette changes the numeric labelling order for the right flanking region nucleotides.

Figure 2-4. Location of primers on the bacterial genome after *sacB-Kan* insertion



3' = Three prime end or tail end

5' = Five prime end or ligation end

2.30 Polymerase chain reaction conditions

Fifty microlitres amplifications were routinely carried out consisting of the components listed in Table 2-11:

Table 2-11. PCR components

Double distilled H_2O	36 µl
10x PCR Buffer	5 µl
10x dNTP mix	5 µl
Primer A 5'	100 pmol in 0.5 µl
Primer B 3'	100 pmol in 0.5 µl
Taq DNA polymerase	2.5 U
Template Zap 198	2 µl

Bacterial DNA templates were derived from boiled lysates of pure cultures of strain *E. coli* O157:H7 ZAP 198.

Reactions were carried out in a pre-heated PCR block (Thermoelectron PX2 Thermal Cycler) at the times and temperatures listed in Table 2-12. Steps 2–4 were repeated 30 times. PCR products were purified using MinElute PCR Purification Kit (Qiagen) as instructed by the manufacturer.

Table 2-12. PCR conditions

Initialisation	94°	4 mins
Denaturation	94°	45 s
Annealing	54–60°	45 s
Elongation	72°	60 s
Final extension	72°	10 mins

2.31 De-phosphorylation of vectors

Plasmid vectors digested with restriction enzymes were treated with shrimp alkaline phosphatase (SAP, Promega) to remove 5' phosphate groups of DNA and aid cloning of inserts into the vector. Restricted vector DNA was incubated at 37° C for 15 mins in 0.1 volume of 10x SAP buffer and 1 unit of SAP (1000 i.u.) followed by 15 mins at 65° C to inactivate the enzyme.

2.32 Agarose gels for DNA

Agarose gels were used to separate and visualise DNA. Gels contained between 0.75 and 2.0% agarose depending on PCR products fragment size. 1 µl of ethidium bromide concentration was added to the gel for visualisation.

2.33 DNA molecular weight markers

To determine the size of the PCR products, one of three molecular weight DNA markers were run: λ *Hind*III marker, 1,000 base and 100 base pair marker (Invitrogen).

2.34 DNA purification from agarose gels

The DNA bands were excised with a scalpel after visualisation on a UV transilluminator (UVP) and extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was reconstituted in 30 µl of dH_2O.

2.35 DNA ligation

Ligations were carried out in dH_2O by adding 5 units of T4 DNA ligase (Biolab) DNA vector (50-100 ng) and 0.1 µl of 10x ligase buffer, in a final volume of 10 µl, followed by overnight incubation at 15° C with an equal molar ratio of DNA insert. The ligation reaction components are presented in Table 2-13.

Table 2-13. Ligation reaction components

1-7 µl	Insert
1 µl	Vector
1 µl	10x Buffer
1 µl	T4 ligase
0-6 µl	dH_2O
10 µl	Total volume

2.36 Topo cloning

The main characteristic of the system is the enzyme DNA topoisomerase, which functions as both restriction enzyme and ligase. The pCR4-TOPO vector was used as indicated in the manufacturer's instructions (Invitrogen). A single 3'-A overhang to each end of the taq-amplified PCR product was added to enable the direct ligation of the homologous regions to the plasmid DNA using TA Cloning (Invitrogen) according to the manufacturer's protocol.

2.37 Preparation of chemically competent cells

A single colony from an LB plate was inoculated into 5 ml of LB and cultured overnight at 37°C with shaking at 200 rpm to an OD_{600} of 0.9, then 100 mls of fresh LB were then inoculated with 1 ml of overnight culture and cultured to an OD_{600} of 0.4-0.6 (approximately 2.5 hours). The suspension was cooled on ice for 30 mins and bacteria harvested by centrifugation for 8 mins at 4,000 rpm in a chilled centrifuge followed by suspension in ice cold TBF1 to a volume 0.4 times that of the initial

culture. The suspension was chilled on ice for a minimum of 15 mins and centrifuged at 4,000 g for 8 mins in a chilled centrifuged. The supernatant was discarded and the bacteria suspended in TBF2 to a volume of 0.02/0.04 of culture and left on ice for 30 mins. Aliquots of the suspension (200 µl) were stored at -70°C.

2.38 Transformation of plasmid DNA by heat shock

10 µl of plasmid DNA was added to 200 µl of previously prepared calcium competent cells and chilled on ice for one hour. Samples were then transferred to a 42°C water bath for 60 s and subsequently chilled on ice for two mins. 800 µl of super optimal broth with catabolite repression was then added and the mix incubated for two hours at 28°C and finally plated onto LBC plates and incubated overnight at 28°C.

2.39 Preparation of electro-competent cells

The final transformation for integration and gene replacement was made with the *E. coli* O157:H7 ZAP 198 strain. Bacteria were cultured overnight at 37°C with shaking at 200 rpm to an OD_{600} of 0.9. 100 mls of LB were inoculated with 1 ml of overnight culture and cultured to an OD_{600} of 0.4-0.6 (approximately 2.5 hours). The suspension was cooled on ice for 30 mins and bacteria harvested by centrifugation for 8 mins at 4,000 rpm in a chilled centrifuge and suspended in 2 ml of ice cold 10% glycerol. 1 µl of purified plasmid was added to a 45 µl aliquot of bacteria, mixed gently and incubated on ice for 5 mins. The mix was transferred to an electroporation cuvette (Flowgen) and electroporated at 2.5 kV. One ml of LB was added and the suspension transferred to an Eppendorf

tube, incubated at 37°C with shaking at 200 rpm for 2 hours and 400 µl plated on LB agar with Chloramphenicol.

2.40 The predicted tertiary structure of OmpC and selection of putative immunogenic peptides

The specific three-dimensional structure of the primary sequence of amino acid molecules that compose OmpC was predicted from a published OmpC *E. coli* O157:H7 sequence (swissprot: locus OMPC_ECO57, accession Q8XE41, Table 2-14) by Swiss-Model (http://swissmodel.expasy.org/), a protein structure homology-modelling server. The tertiary structure was visualised with DeepView-Swiss-PdbViewer 3.5 (Figure 2-5). Comparison of the predicted sequence was made with recent published sequences of known structures in the Protein Data Bank (Basle et al. 2006).

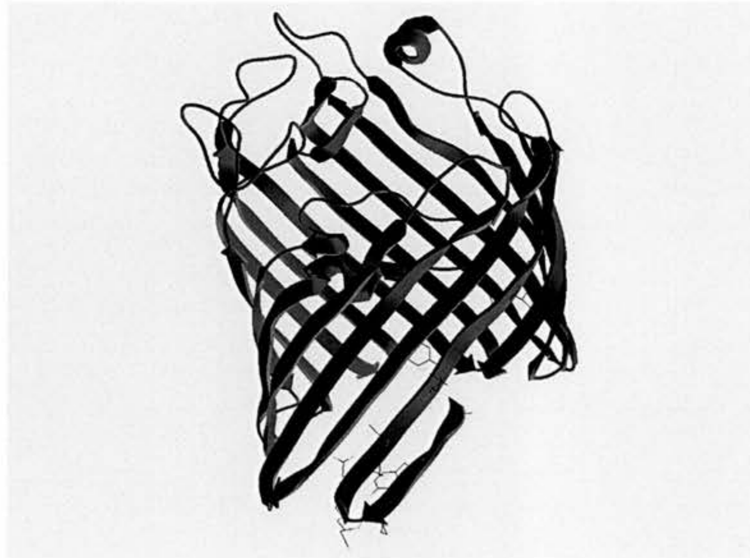
Table 2-14. *E. coli* O157:H7 EDL933 amino acid OmpC sequence.

In grey precursor protein, underlined amino acid sequences of five external loops.

```
1    MKVKVLSLLV PALLVAGAAN AAEVYNKDGN KLDLYGKVDG LHYFSDDKSV DGDQTYMRLG
61   FKGETQVTDQ LTGYGQWEYQ IQGNSAENEN NSWTRVAFAG LKFQDVGSFD YGRNYGVVYD
121  VTSWTDVLPE FGGDTYGSDN FMQQRGNGFA TYRNTDFFGL VDGLNFAVQY QGKNGSVSGE
181  GMTNNGREAL RQNGDGVGGS ITYDYEGFGI GAAVSSSKRT DDQNSPLYIG NGDRAETYTG
241  GLKYDANNIY LAAQYTQTYN ATRVGSLGWA NKAQNFEAVA QYQFDFGLRP SLAYLQSKGK
301  NLGVINGRNY DDEDILKYVD VGATYYFNKN MSTYVDYKIN LLDDNQFTRD AGINTDNIVA
361  LGLVYQF
```

Perna et al., 2001

Figure 2-5. OmpC hollow B-barrel fold. Image produced with Swiss-Pdb viewer 3.5



2.41 Antibiotics

The list of antibiotics used during this work is presented in Table 2-15.

Table 2-15. Antibiotics

Antibiotic	Solvent	Stock concentration	Final concentration
Ampicillin	dH_2O	10 mg ml^{-1}	50 µg ml^{-1}
Chloramphenicol	Ethanol	25 ml^{-1}	25 µg ml^{-1}
Kanamycin	dH_2O	10 ml^{-1}	50 µg ml^{-1}

2.42 Creation of the *ompC* deletion

The allelic exchange method was used to replace the *ompC* gene with a *sacB-kan* marker cassette in *E. coli* O157:H7 ZAP198 to generate strain *E. coli* O157:H7 ZAP 1187. This technique has proved very successful in

avoiding unwanted genetic rearrangements in "wild type" strains such as *E. coli* O157:H7 (Emmerson et al. 2006). The exchange process relies on the steps described in Table 2-16.

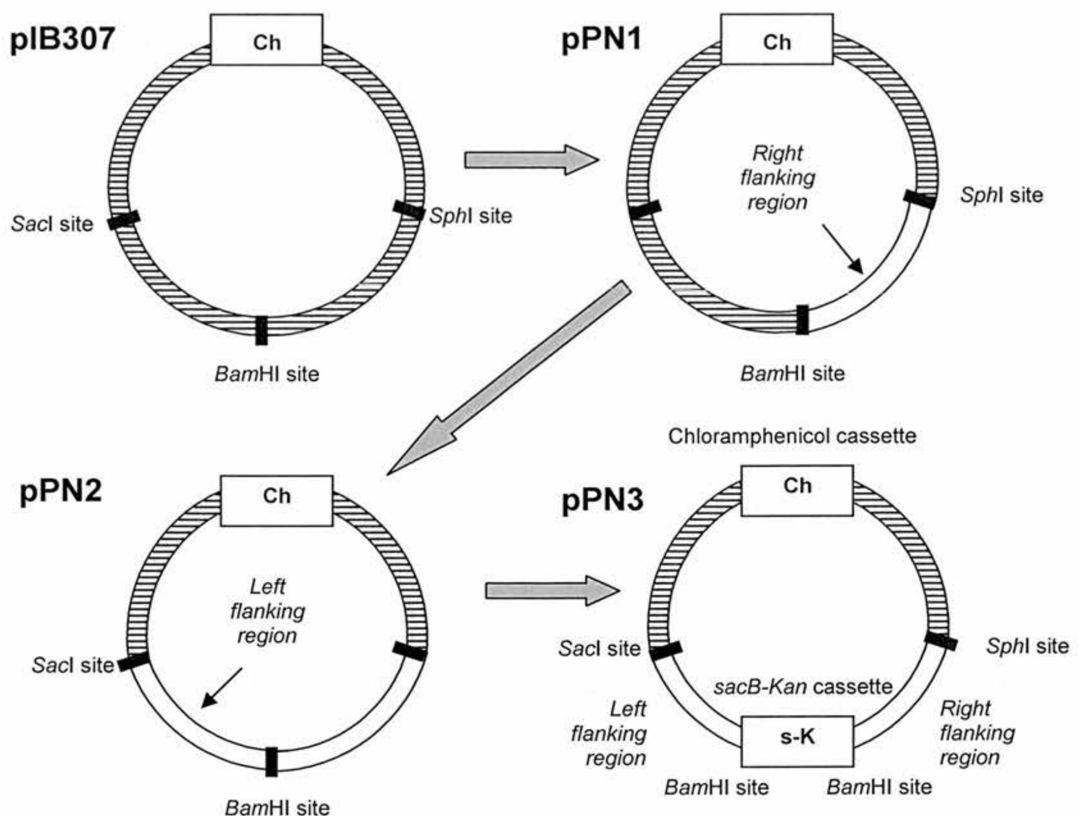
Table 2-16. Outline of the allelic exchange method

Construction of a Plasmid vector	Two regions homologous to the flanking regions of the target gene and a *sacB-kan* cassette are cloned into the original temperature sensitive (28°C), chloramphenicol resistant plasmid.
Transformation	Introduction of the plasmid into electro-competent *E. coli* O157:H7 receptor strain
Plasmid integration and first recombination	Bacteria are grown at a temperature (42°C) that precludes plasmid replication, while the use of Chloramphenicol rich media (LBC) selects resistant bacteria in which the plasmid has been integrated by recombination of homologous regions into the bacterial genome.
Plasmid excision and second recombination	Bacteria are cultured at 28°C in Kanamycin to induce plasmid excision. A second round of allelic exchange between the plasmid and the bacterial genome generates a plasmid containing the original bacterial gene and flanking regions, and a bacteria with the plasmid homologous regions and marker cassette.
Plasmid curing and selection of the mutant	Bacteria are replica-plated in Kanamycin (LBK) and LBC rich media. Bacteria that grow exclusively on LBK have been successfully exchanged.

The allelic exchange approach is based on the use of a temperature sensitive plasmid vector containing a genetic marker that confers a phenotype that can be selected for, and a multiple cloning site containing several restriction sites, to allow the insertion of DNA fragments at these locations. In this work we used plasmid pIB307, that codes for chloramphenicol resistance and is unable to replicate autonomously over 28°C. In addition, this plasmid was further modified by the insertion of two regions of approximately 1,000 bp, that are the amplified flanking regions of the bacterial *ompC* gene targeted for replacement.

These homologous regions are extended at each end by the addition of sequences recognised by specific restriction enzymes, to allow their insertion into the complementary fragments present in the original plasmid vector (*SacI* 1,484, *Bam*HI 1,495 and *SphI* 1,523 bp). For this project, the right flanking region was amplified with the addition of *SacI* (5') and BamHI (3') sites, and cloned into pIB307 creating pPN1. The left flanking region incorporated *Bam*HI (5') and *SphI* (3') sites and was inserted into pPN1 forming pPN2. Finally, the *sacB-kan* cassette was inserted between both flanking regions at the *BamHI* site to create construct pPN3. The diagram is represented in the scheme depicted on Figure 2-6.

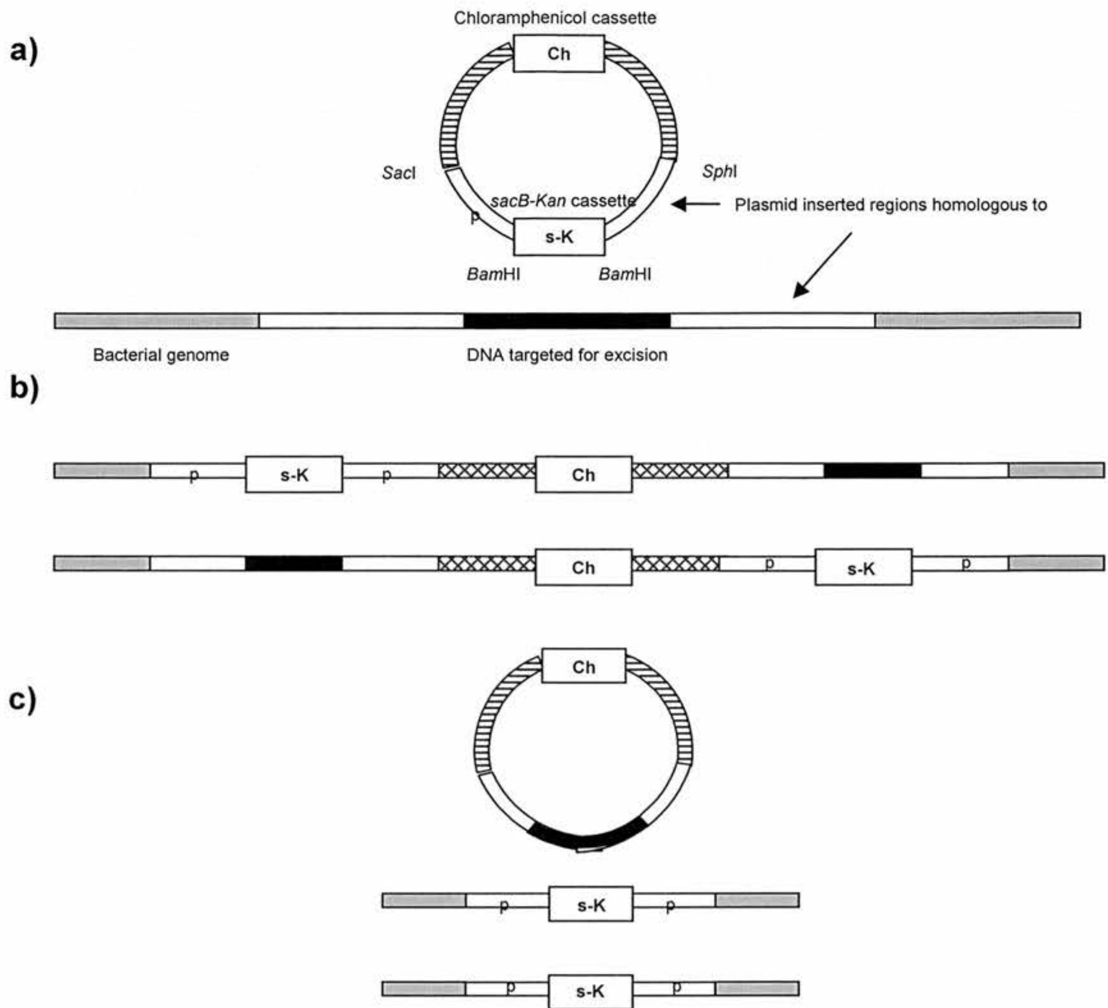
Figure 2-6. Plasmid construction



This construct was then used to exchange the target gene and its flanking regions by the plasmid homologous flanking regions and cassette. The *kan* gene is inserted in the bacterial genome to facilitate the selection of a kanamycin-resistant transformants lacking the replacement gene. The *sacB* helps to identify subsequent excisions of the marker cassette if required.

Plasmid pPN3 containing the *ompC* flanking regions and the marker *sacB-Kan* and Chloramphenicol resistance cassettes was electroporated into *E. coli* O157:H7 ZAP198 (Figure 2-7a). Resultant transformants were selected on LB (Luria–Bertani) containing chloramphenicol (LBC, 25 µg/ml) and incubated at 28°C. Resistant colonies were grown in LBC broth (Luria-Bertani broth containing 25 µg/ml of chloramphenicol) at 42°C and subcultured three times more for 12 hours to obtain primary plasmid integrates (Figure 2-7b). Further sub-culturing into LBK (Luria-Bertani broth containing 50 µg/ml of kanamycin) at 28 °C selected for plasmid excision where the *ompC* gene had been replaced by the *sacB-kan* marker cassette (Figure 2-7c).

Figure 2-7. Replacement of the *ompC* gene by the *sacB-Kan* cassette



Finally, the region exchanged is PCR amplified. The construct is validated by verifying the loss of the plasmid to determine: (i) absence of *ompC* (ii) presence of the *sacB-kan* cassette and (iii) correct location of the insert.

Chapter 3

Enumeration of *E.coli* O157:H7 in gastrointestinal tract tissues, contents and faeces following experimental challenge of cattle via oral and rectal routes

3.1 Introduction

It is important to understand the shedding patterns of *E. coli* O157:H7 to identify possible intervention points that may decrease its prevalence in cattle. The study of cattle shedding patterns could also help to prevent human disease, in particular if we consider that high level excretion may be a major risk factor for human exposure to this pathogen (Ogden et al. 2004; Omisakin et al., 2003).

Shedding of *E. coli* O157:H7 from cattle has been frequently reported as intermittent (Brown et al., 1997; Cray Jr. and Moon, 1995; Garber et al., 1995; Liu et al., 2005; Pearce et al., 2004a; Robinson et al., 2004) and on certain occasions the same strain has been detected in the same animal weeks apart (Liu et al., 2005; Pearce et al., 2004a). The concentration at which *E. coli* O157:H7 is shed in faeces varies from animal to animal and levels of up to 10^5-10^6 CFU g^{-1} of wet faeces have been reported regularly (Brown et al., 1997; Cray Jr. and Moon, 1995; Matthews et al., 2006b; Omisakin et al., 2003). The duration of faecal shedding has been estimated for experimental and naturally infected animals between 1 to 12 weeks (Cray Jr. and Moon, 1995; Rahn et al., 1997; Robins-Browne and Hartland, 2002; Sanderson et al., 1999; Widiasih et al., 2004b) with isolated individuals capable of sustaining high shedding levels for even longer periods (Brown et al., 1997; Ohya et al., 2000). It is generally agreed that weaned calves are more susceptible to colonisation and that they shed *E. coli* O157:H7 in their faeces with greater magnitude and intensity than older cattle (Gannon et al., 2002; Garber et al., 1995; Hancock et al., 1997; Nielsen et al., 2002; Rugbjerg et al., 2003). Besides

age, a varied complexity of often-interrelated microbial, animal, herd, environmental and production factors are known to influence to a lesser extent bacterial shedding (Fitzgerald et al., 2003; Minihan et al., 2003; Ogden et al., 2004; Van Baale et al., 2004).

Initial research aimed at identifying colonisation sites of naturally and experimentally infected cattle, has reported the isolation of *E. coli* O157:H7 from all areas of the gastrointestinal tract including occasionally the tonsils and gall bladder (Brown et al., 1997; Cray Jr. and Moon, 1995; Grauke et al., 2002; Jeong et al., 2007; Naylor et al., 2003a; Stoffregen et al., 2004). Cray Jr. and Moon (1995) reported increased numbers of *E. coli* O157:H7 from caecal contents to faeces. Brown *et al.* (1997) reported the highest recovery rate in the contents of the forestomachs. Stoffregen *et al.*(2004) and Jeong *et al.* (2007) cultured the bacteria from rumen, large colon contents and bile of experimental and naturally colonised animals. Grauke *et al.*(2002) using ruminal and duodenal cannulae to sample live animals did not detect, after 34 days, any bacteria in these areas of the gastrointestinal tract despite animals shedding *E. coli* O157:H7 in their faeces. The subsequent finding that *E. coli* O157:H7 colonises primarily the rectal anal junction of cattle focused research on this site (Naylor et al., 2003a).

3.2 Aims and objectives

1- To confirm in a greater number of animals the rectal tropism of *E.coli* O157:H7 as originally reported.

2- To compare the similarities and efficiency of rectal inoculation versus oral challenge in establishing *E. coli* O157:H7 infection.

3- To investigate the possible colonisation of other sites situated rostraly to the rectum in the intestinal tract, and any possible relevance for sustaining infection.

To achieve these objectives the following approaches were taken:

Daily collection of the faeces from 54 challenged calves, for a minimum of two weeks, followed by detection and enumeration of total bacteria in all the samples.

A total of 22 animals were examined *post-mortem* and various tissue and contents samples collected from the gastrointestinal tract for *E. coli* O157:H7 bacterial counts.

3.3 Results

A total of 54 animals (Group 1, section 2.2 Material and Methods, M&M) were challenged with *E. coli* O157:H7 either by oral administration or by direct application of the organism to the recto-anal junction. From these a total of 46 animals became colonised and a *post-mortem* was carried out on 22 animals still shedding greater than 1 x 10^1 CFU g^{-1} of *E. coli* O157:H7 beyond day 14 post-challenge. Ten of these animals had been challenged by direct rectal application and 12 by oral administration. Initially six animals were cannulated and ruminally challenged. The bacterial colonisation rates and persistence did not differ from the group of orally challenged animals and this particular route was discontinued. In this analysis, the data from ruminally challenged animals has been included in the orally challenged group.

3.3.1 Shedding analysis

Faeces were obtained from each animal following stimulation of the anal sphincter and separated into core and surface components. This was a critical step in the bacterial detection system so that for each faecal sample the surface and core samples could be cultured independently, as it was assumed to be an indication of whether the majority of bacterial contamination was arising locally at the rectum and terminal areas of the distal colon (surface) or from higher sites in the GIT (core).

Arbitrary criteria were developed to categorise the experimental faecal shedding data. Calves were classified as colonised when the bacterial numbers were above 10^3 CFU g^{-1} of *E. coli* O157:H7 at day five. Persistent

infection was when the shedding levels, for more than 50 % of samples, were above 10^3 CFU g^{-1} of *E. coli* O157:H7, between days 5 and 14 and still detectable at day 14 of those with successful uptake. Animals were classified as specifically rectally colonised if the faecal surface counts were at least 10 fold higher than in the core samples for more than 50% of occasions between days 5 and 14. The entire data set and descriptive statistics are presented in Annex Tables I and II.

In this categorisation from the 54 animals challenged, 22 out of 24 (91.7%) rectally challenged and 20 out of 30 (66.7%) calves either orally or intraruminally challenged were considered successfully colonised. *E. coli* O157:H7 was detected for at least 14 days in 15 of 22 (68.2 %) in the faeces of rectally challenged animals and 13 of 20 (65.0%) orally challenged animals. Of these animals rectal colonisation was considered established in 15 out of 15 (100.0%) rectally challenged and 11 of 13 (84.6.0%) orally challenged cases. Overall 77.8 % of 54 animals become colonised, 66.7 % of the 42 shed the bacterium for at least 14 days, and 92.9 % of 28 were colonised by *E. coli* O157:H7 in the terminal rectum (Table 3-1). Additionally, case 684, which had been orally challenged, did colonise and could have been considered rectally colonised, but it only started shedding levels above 10^3 CFU g^{-1} of *E. coli* O157:H7 from day 13 to 21, and therefore it had to be categorised as non-colonised.

Table 3-1. Analysis of *E. coli* O157 colonisation, persistence and rectal localisation in experimentally colonised calves

	Rectal		%	Oral		%	Total
Colonised	22	24	**91.7 %**	20	30	**66.7 %**	**77.8 %**
Persistence	15	22	**68.2 %**	13	20	**65.0 %**	**66.7 %**
Rectal Colonisation	15	15	**100 %**	11	13	**84.6 %**	**92.9 %**

Colonised: Shedding levels above 10^3 at day five, 10 fold or higher levels in surface than core or persistence until day 9

Persistence: Shedding levels consistently (> 50 % of samples) above 10^3 between days 5 and 14 and still detectable at day 14 of those with successful uptake.

Rectal Colonisation: Surface at least 10 fold higher than core for > 50% of samples between days 5 and 14

After the initial two days the shedding patterns with significantly higher numbers (paired Student's T-test, $p<0.001$) present in surface samples compared to core were seen for the two different challenge routes (Figures 3-1 and 3-2). Interestingly, the faecal *E. coli* O157:H7 counts for the first two days were similar in core and surface samples for orally challenged calves. In rectally challenged animals, higher numbers of *E. coli* O157:H7 CFU g^{-1} were immediately detected in surface samples.

Figure 3-1. Orally challenged calves. Daily group 1 mean of faeces surface and core *E. coli* O157:H7 levels

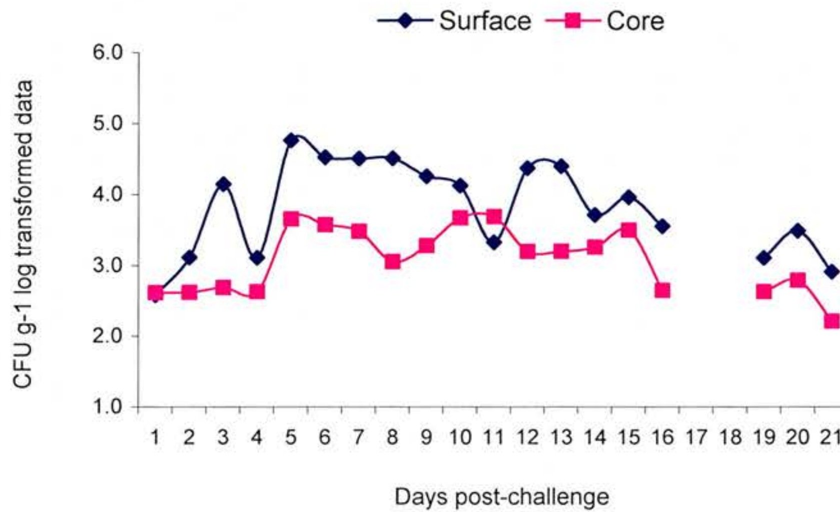
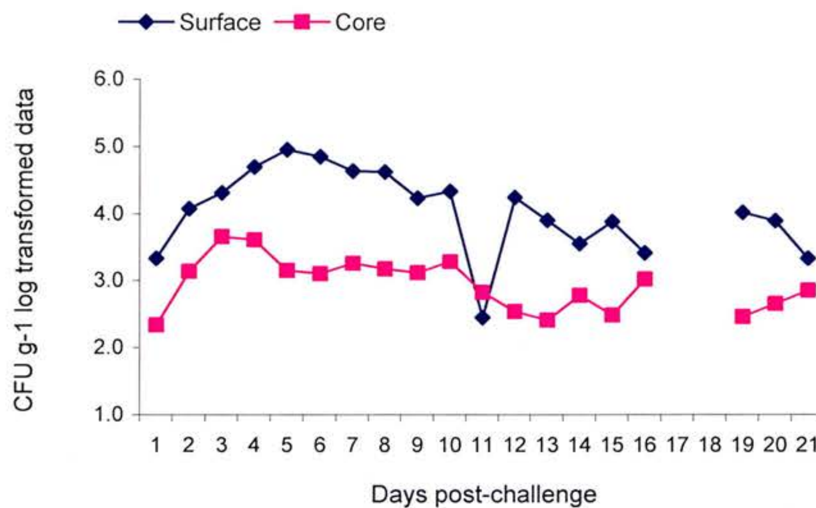
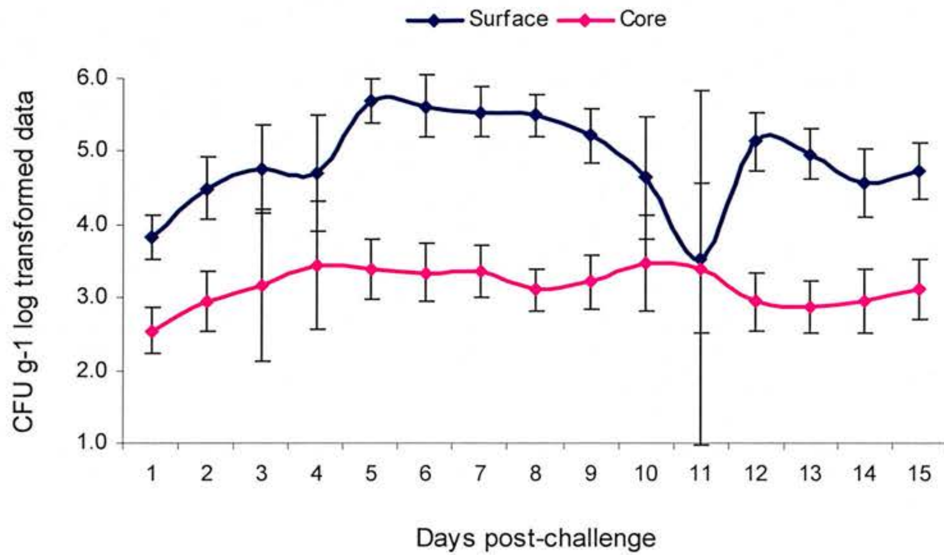


Figure 3-2. Rectally challenged calves. Daily group 1 mean of faeces surface and core *E. coli* O157:H7 levels



Highly statistically significant differences ($p<0.001$) were found between surface and core counts for the whole dataset, and for the log-transformed subset formed after excluding zero values, by two way Student T-test respectively (Figure 3-3).

Figure 3-3. Orally and rectally challenged animals. Daily group 1 mean of faeces surface and core *E. coli* O157:H7 levels



No statistically significant differences were found for surface or core values, when the average daily shedding of orally challenged animals was compared with animals that were administered bacteria rectally ($p>0.14$ and $p>0.15$ respectively) (Figures 3-4 and 3-5).

Figure 3-4. Orally versus rectally challenged animals. Daily group 1 mean of faeces surface *E. coli* O157:H7 levels

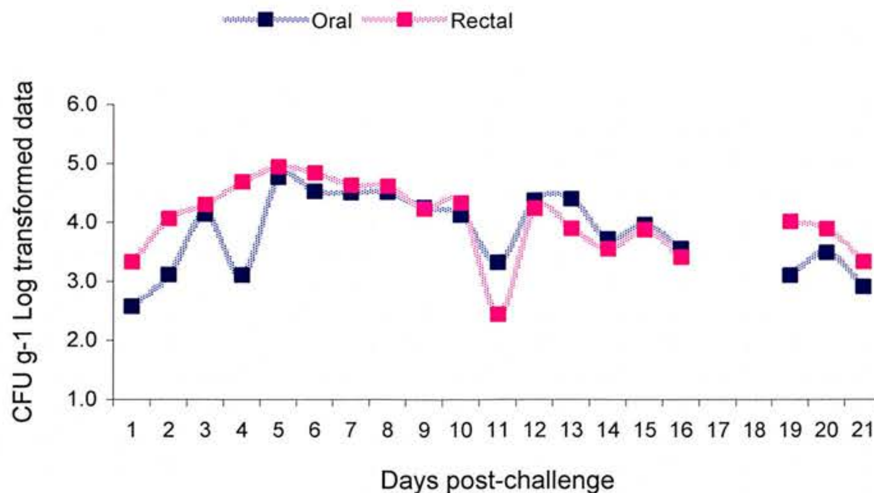
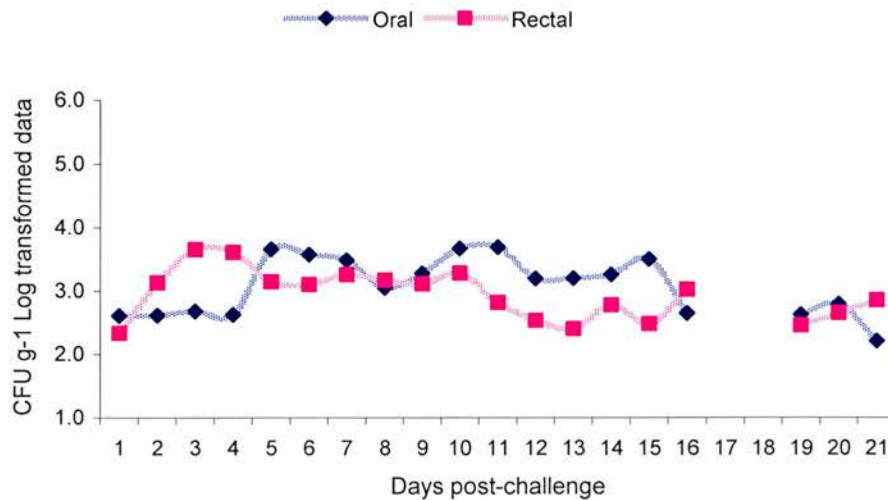


Figure 3-5. Orally versus rectally challenged animals. Daily group 1 mean of faeces core *E. coli* O157:H7 levels



It is notable that, unlike the surface, the frequency distribution of core CFU g^{-1} of *E. coli* O157:H7 values in colonised animals is bimodal, with the highest frequency at the zero interval and the second mode with 10^2 CFU g^{-1} of *E. coli* O157:H7 (Figures 3-6 and 3-7). Thus, the probability of the core being negative is significantly higher than in the surface (X^2, $p<0.001$).

Figure 3-6. Frequency distribution of *E. coli* O157:H7 levels on the surface of the faeces from colonised group 1 animals

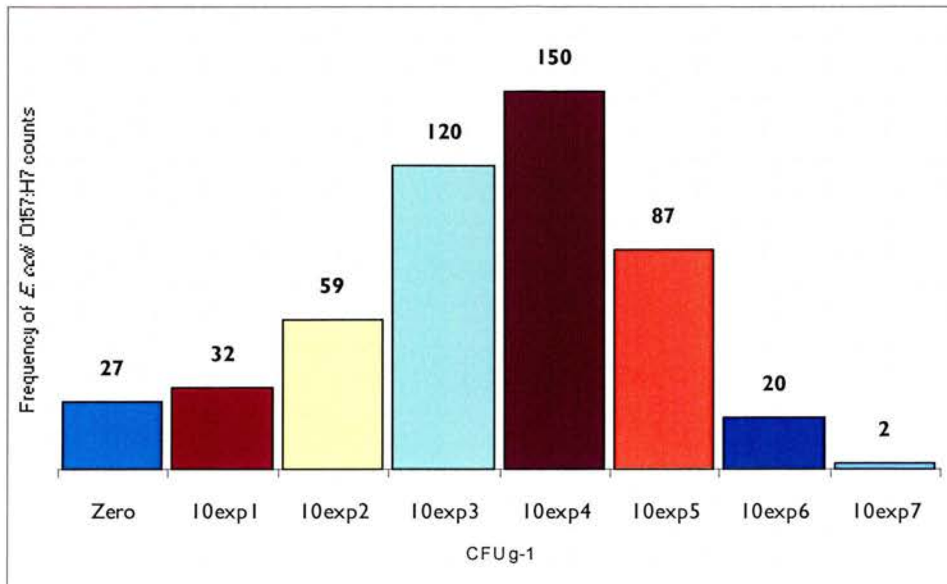
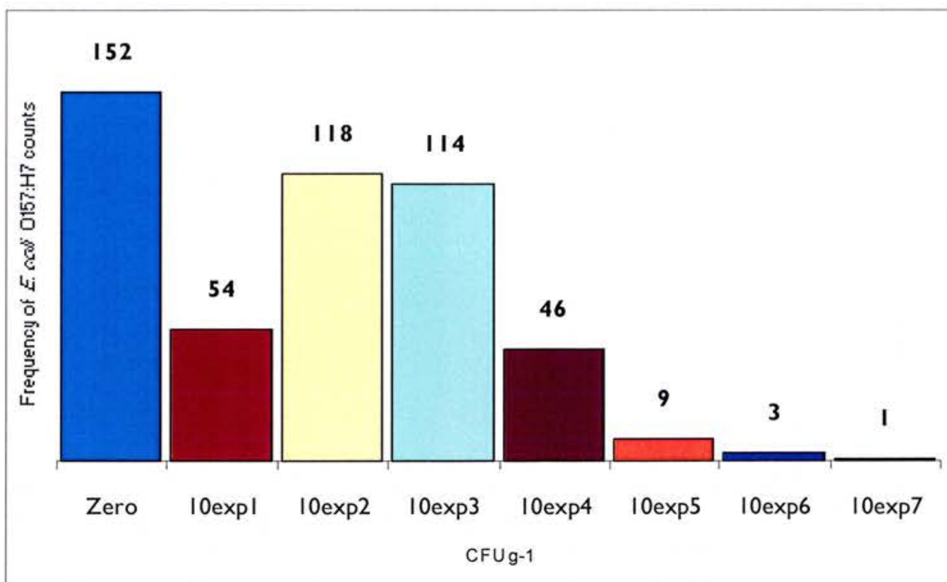


Figure 3-7. Frequency distribution of *E. coli* O157:H7 levels in the core of the faeces from colonised group 1 animals



3.3.2 Post-mortem results

Overall *E. coli* O157:H7 was present in 119 samples from contents or tissue washings. On 54 occasions, samples of tissue and contents were taken from the same gastrointestinal sites. On 25 occasions *E. coli* O157:H7 was detected in both contents and tissue washings taken from the same site (agreement in 46.3% of the samples, Kappa value 0.4, $p<0.001$). Bacteria were exclusively found in contents in 16 samples and only in tissues in 13 cases.

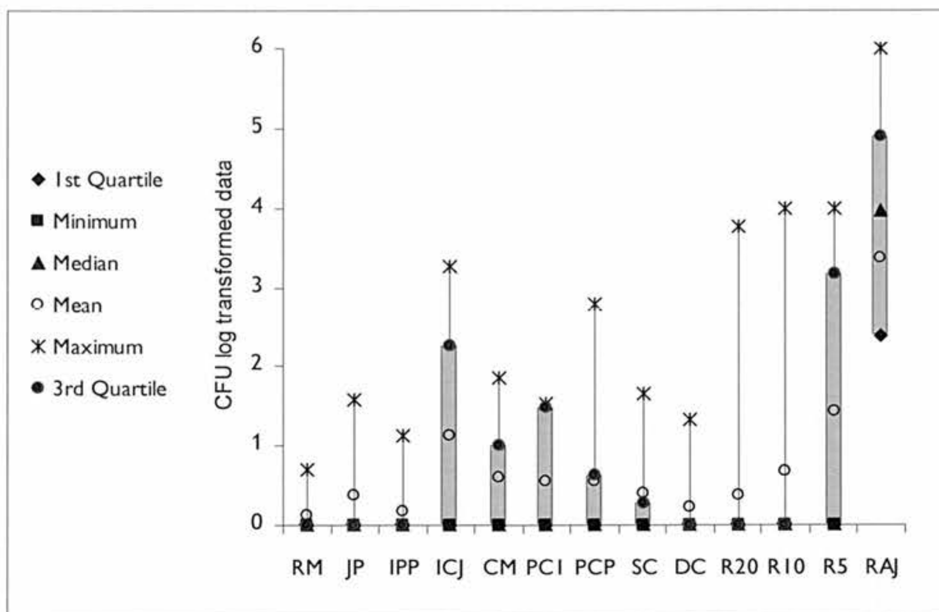
In the 22 animals still shedding the organism two weeks following challenge and that were examined by *post mortem*, *E. coli* O157:H7 was detected in the rectum in 21 out of 22 (95.5%) tissue washes from the terminal rectum (TR) and in 15 of 21 (71.4%) from the area 5 centimetres proximally. For the other rectal areas, 6 out of 21 and 7 out of 22 samples were positive at either 10 or 20 centimetres (28.6% and 31.8% respectively). Results for all animals are summarised in Annex Table III.

In the orally challenged group, *E. coli* O157:H7 was isolated from washings of the recto-anal junction (RAJ) and at concentrations up to 10^6 CFU cm^{-2} in 11 of the 12 animals (Figure 3-8). A proportion of the gastrointestinal mucosal washings collected from the large intestine contained *E. coli* O157:H7 but the concentrations were several logarithm ranges lower than from the corresponding rectal mucosal samples except for case 691. In total, 5 individuals out of 12 had positive large intestine tissue washings at concentrations of up to 10^3 CFU cm^{-2} of *E. coli* O157:H7. Significant ($p=0.009$) high values in non-rectal tissue were obtained from mucosal washings of the ileo-caecal valve lymphoid patch

when compared with the rest of the counts obtained from the rest of the colon, with the highest concentration of *E. coli* O157:H7 obtained for one individual at 10^3 CFU cm^{-2}.

The statistical analysis of data showed that *E. coli* O157:H7 counts from tissue washings of the terminal rectum were significantly higher than from tissues of the large colon (P=0.001). Significantly higher counts (P<0.001) were detected in the terminal rectum when compared to counts from tissue washings of the rest of the rectum (5, 10 and 20 cm distal to the recto-anal junction).

Figure 3-8. Orally challenged animals. Statistical analysis of *E. coli* O157:H7 levels from gastrointestinal mucosa washings

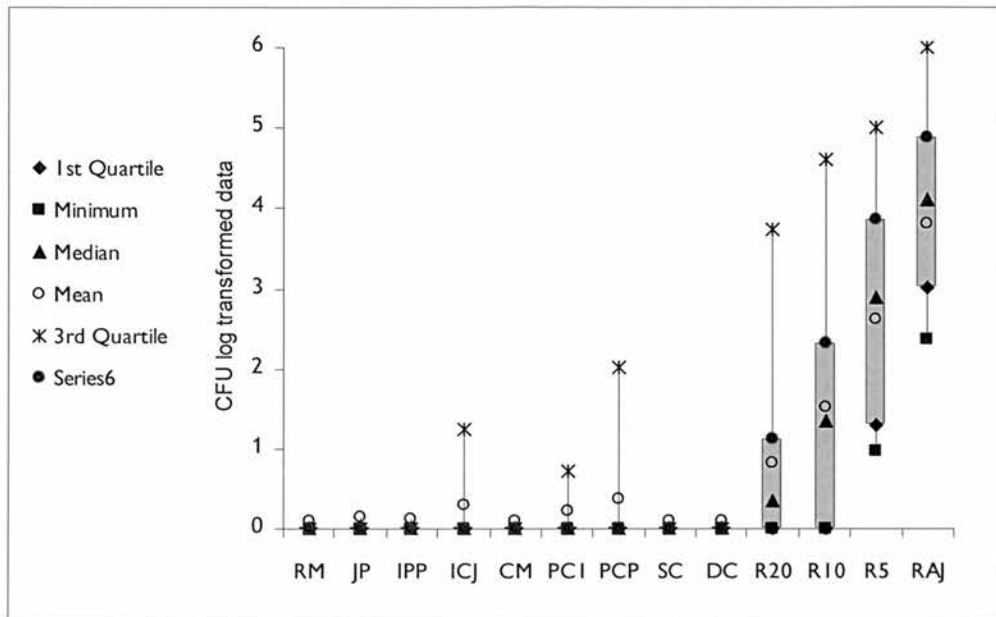


RM	Rumen	SC	Spiral colon
JP	Jejunum	DC	Distal colon
IPP	Ileum	R20	Rectum (+) 20
ICJ	Ileo caecal junction	R10	Rectum (+) 10
CM	Caecum	R5	Rectum (+) 5
PC	Proximal colon	TR	Terminal Rectum
PCP	Proximal colon patch		

In the calves challenged by direct rectal application *E. coli* O157:H7 was identified from mucosal tissue washings taken at the RAJ in all animals at concentrations up to 10^6 CFU cm^{-2} (Figure 3-9). The *E. coli* O157:H7 colonisation extended proximally in the rectum in the highest shedding individuals and sites 10 cm and 20 cm proximal to the RAJ were positive in 50% of individuals. Mucosal washings were occasionally positive for large intestinal sites but never in more than two individuals and consistently at concentrations lower than 10^2 CFU cm^{-2} *E. coli* O157:H7.

For animals that were challenged *per rectum*, the statistical analysis of data showed that counts from tissue washings of the terminal rectum were significantly higher than from tissues of the large intestine ($P<0.001$). Significantly higher counts ($P<0.001$) were detected in the terminal rectum when compared to counts from tissue washings of the rest of the rectum (areas comprising 5, 10 and 20 cm from the terminal rectum).

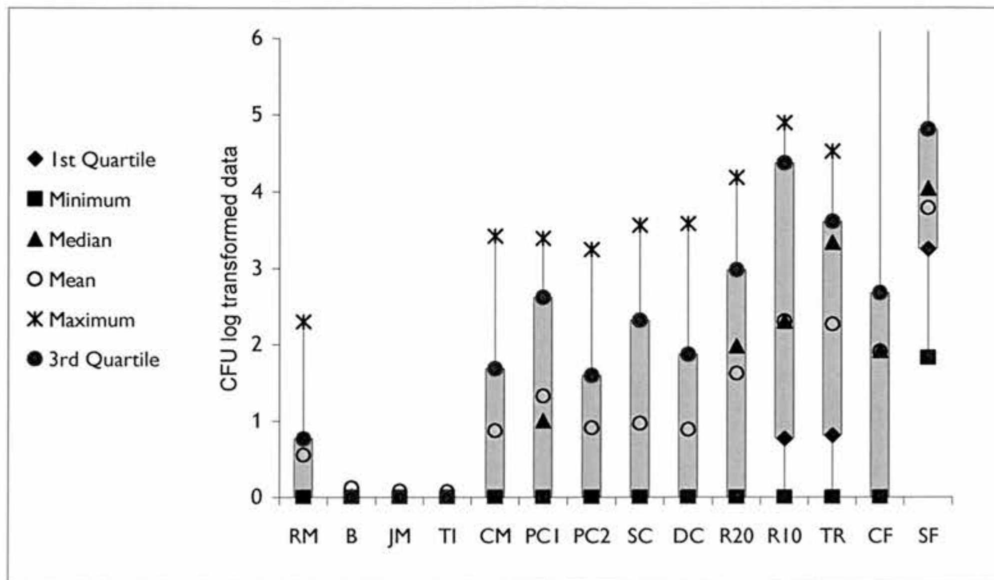
Figure 3-9. Rectally challenged animals. Statistical analysis of *E. coli* O157:H7 levels from gastrointestinal mucosa washings



RM	Rumen	SC	Spiral colon
JP	Jejunum	DC	Distal colon
IPP	Ileum	R20	Rectum (+) 20
ICJ	Ileo caecal junction	R10	Rectum (+) 10
CM	Caecum	R5	Rectum (+) 5
PC	Proximal colon	TR	Terminal Rectum
PCP	Proximal colon patch		

Results of *E. coli* O157:H7 counts from rectal contents taken *post-mortem* in orally challenged calves were statistically significantly lower compared to same day faecal counts (p=0.004). In contents samples, highly significant lower bacterial counts were detected in the large intestine than those in the rectum (P=0.004). Significant statistical differences were not detected between faeces collected at the terminal rectum and other areas of the rectum (p=0.610, Figure 3-10)

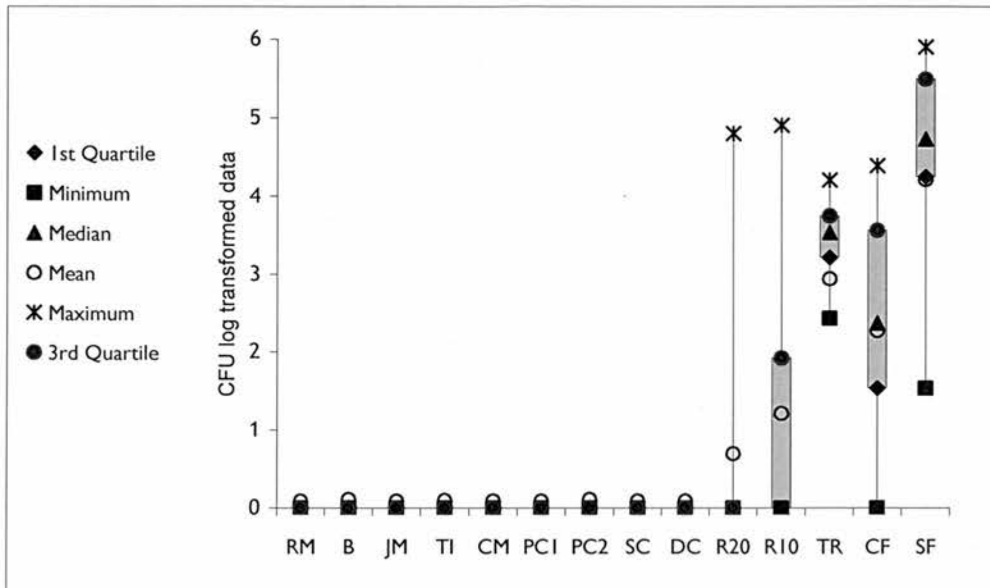
Figure 3-10. Orally challenged animals. Statistical analysis of *E. coli* O157:H7 levels from gastrointestinal contents



RM	Rumen	SC	Spiral colon
B	Bile	DC	Distal colon
JM	Jejunum	R20	Rectum (+) 20
TI	Terminal Ileum	R10	Rectum (+) 10
CM	Caecum	TR	Terminal Rectum
PC1	Proximal colon at Ileocaecal junction	CF	Faecal core
PC2	Proximal colon at proximal lymphoid patch	SF	Faecal surface

In animals that were challenged *per rectum*, significant lower values were found in terminal rectum contents compared to same day surface faecal counts ($p<0.001$), while significantly lower values ($p<0.001$) were detected in the the large intestine compared to the rest of the rectum. A significant p value of 0.011 was found for the higher *E. coli* O157:H7 numbers at terminal rectum compared to the rest of the rectum (Figure 3-11).

Figure 3-11. Rectally challenged animals. Statistical analysis of *E. coli* O157:H7 levels from gastrointestinal contents



RM	Rumen	SC	Spiral colon
B	Bile	DC	Distal colon
JM	Jejunum	R20	Rectum (+) 20
TI	Terminal Ileum	R10	Rectum (+) 10
CM	Caecum	TR	Terminal Rectum
PC1	Proximal colon at Ilocaecal junction	CF	Faecal core
PC2	Proximal colon at proximal lymphoid patch	SF	Faecal surface

In both orally and rectally challenged animals the faecal surface samples were always positive for *E. coli* O157:H7 with concentrations over 10^4 CFU g^{-1} in 13 of the 21 collected samples. For the animals challenged by direct rectal administration, *E. coli* O157:H7 was not recovered from bile or samples of digesta from non-rectal sites that included rumen, small or large intestine. For the orally challenged group, *E. coli* O157:H7 was not recovered from bile. Although, samples of digesta from large intestinal sites were occasionally positive the *E. coli* O157:H7 concentrations were always lower than from rectal or surface faecal samples.

Discussion

A marked bacterial tropism for the terminal rectum in the bovine host has been confirmed. For all cases, *E. coli* O157:H7 principally colonised the tissues of the last three centimetres of the terminal rectum. In the contents, the highest numbers of *E. coli* O157:H7 were also detected from samples obtained from the last few centimetres of the rectum and greater numbers were detected from the faecal surface after transit through the terminal rectum.

Other minor sites were identified. These included the rumen, small intestine and more frequently the proximal colon, in particular at the ileo caecal junction. Nevertheless, the bacterial numbers were significantly lower in tissues than in the ingesta. This was true even for proximal rectum tissues compared to the terminal rectum. In two animals out of the 24 orally challenged animals studied *E. coli* O157:H7 was distributed throughout the large intestine and was not localised specifically at the rectal mucosa given the even distribution of the bacteria throughout the faecal pat. This finding is consistent with previous reports (Naylor et al., 2003a) and suggests that there is a different mechanism of colonisation for a small number of animals. It is apparent that the colonisation of other sites situated higher up in the intestinal tract may occur and its relevance for sustaining infection has not been yet analysed. Although, for the period of three weeks analysed in this study the patterns of shedding were undistinguishable for animals orally and rectally challenged, suggesting that colonisation of sites higher up in the large

intestine do not play a determinant role in the magnitude or duration of infection.

Several reasons could account for the reported sporadic shedding described by many authors: it may be genuine, induced by repeated reinfection, or influenced by factors that may alter detection capacity affecting both magnitude and prevalence estimates. Factors that may affect carriage estimates at an individual level are: differences in detection techniques, sensitivity of tests, or the type of sample used (entire faeces, faecal pat, faecal or oral swab (Pearce et al., 2004b; Rice et al., 2003; Sanderson et al., 1995; Stanford et al., 2005). The significant difference of bacterial counts found on the surface when compared to the core of the faeces is also indicative that the contamination occurs at the point where the intestinal contents move through the rectum. This finding may explain the peculiar bimodal distribution of *E. coli* O157:H7 CFU g^{-1} frequencies found for faecal cores. The first peak is at zero, and could correspond to the true central tendency of the core values. The source of the second peak, at $4x10^2$ CFU g^{-1} of *E. coli* O157:H7 may be a result of contamination, resulting from the difficulties of obtaining totally uncontaminated core for all samples given the varied shape and consistency of the faeces. Furthermore, in colonised animals only 5.4% of the samples were *E. coli* O157:H7 negative on the surface compared with 30.6% negative in the core. Thus, sampling accuracy improves considerably when faeces are sampled on the surface. The characteristic distribution of *E. coli* O157:H7 predominantly on the surface of the faeces may explain the widely reported intermittent individual shedding in cattle (Robinson et al., 2004; Stanford et al., 2005). In most of the studies

faeces have been collected either directly from the ground or from the rectum and therefore not taking into consideration the specific distribution of *E. coli* over the faecal surface induced after final passage through the terminal rectum.

This work also demonstrates that the experimental rectal challenge with *E. coli* O157:H7 is indistinguishable, with respect to the shedding patterns, when compared with the natural oral route. Animals rectally or orally challenged showed identical *E. coli* O157:H7 shedding patterns. There was no difference in average daily shedding, rectal colonisation rates or bacterial distribution on the surface and core of the faeces, which was highly statistically significant in this study and in previous work undertaken by our group (Naylor et al., 2003b). Importantly the rectal inoculation was more effective in establishing infection and subsequent rectal colonisation. The efficacy of the rectal challenge to establish infection is supported by another recent study where all animals challenged rectally were colonised (Sheng et al., 2004), thus supporting initial evidence identifying the terminal rectum as the principal site for *E. coli* O157:H7 colonisation (Naylor et al., 2003b).

The highest numbers of *E. coli* O157:H7 in the intestinal contents were detected in the rectum. Remarkably, a statistically significant increase was detected in the surface of the faeces collected a few hours before the necropsy when compared with rectal contents obtained inside the rectum and before excretion. This finding suggests that the large intestine discharge reaches its maximal contamination only after contacting the epithelium of the terminal 2/3 centimetres of the rectum.

The post-mortem examination of these colonised animals also allowed the identification of minor sites of *E. coli* O157:H7 carriage. These sites included the rumen, small intestine and most frequently, the proximal colon, particularly at the ileo caecal junction. In the large colon, no clear association was found between areas rich in lymphoid tissue and *E. coli* O157:H7, a finding corroborated by a recent study on long-duration culture-positive cattle (Lim et al., 2007). In this study, in all but one case, *E. coli* O157:H7 principally colonised the tissues of the last three centimetres of the terminal rectum, while it was present less frequently and in lower numbers, as the samples were taken further away from the terminal rectum. Support of the importance of terminal rectum colonisation also comes by a recent abattoir study carried out in naturally colonised animals where the terminal rectum was detected as the principal site of colonisation in 15 out of 16 *E. coli* O157:H7 positive animals (Low et al., 2005).

This study confirms the terminal rectum tropism of *E. coli* O157:H7 and clarifies the importance of different gastrointestinal areas for the colonisation and persistence of the bacterium. The ability of bacteria to colonise gastrointestinal tissues depends on its capacity to adhere or invade host epithelia. These bacterial characteristics are of major importance in enteric infections and provide adequate grounds for the investigations of the interactions of bacteria with the gastrointestinal epithelium on a microscopic scale.

Chapter 4

Histological and ultrastructural study of *E. coli* O157 colonisation of the terminal rectum

4.1 Introduction

Although enterohaemorraghic *E. coli* O157:H7 causes no apparent disease in cattle (Caprioli et al., 2005; Naylor et al., 2005a), epithelial attachment of *E. coli* O157:H7 is unlikely to be a neutral process. From an understanding of the area of the gastrointestinal tract that *E. coli* O157:H7 colonises, there is an opportunity to examine the interaction at the terminal rectum to determine whether there is evidence of an immune response to colonisation.

A feature of all typical EHEC strains is their close association to intestinal epithelial cells where the bacterium causes rearrangement of enterocyte actin cytoskeleton fibres to form pedestal like structures that elevate the bacteria above surrounding cell surfaces (Roe et al., 2003). This intimate bacterial association with the host induces important alterations of the intestinal epithelium, which are potential triggers of local inflammatory reactions mediated through the interaction of pathogen-associated molecular patterns and host pattern recognition receptors (Magalhaes et al., 2007). Intestinal epithelial cells are also capable of expressing major histocompatibility complex type II molecules and have also been involved in antigen presentation (Mallegol et al., 2005; Van Niel et al., 2003).

To date the only reported immune reactions in the intestinal tract to *E. coli* O157:H7 colonisation have been described for experimental infections of gnotobiotic, neonatal or immunosuppressed calves and in sites other than the rectum (Dean-Nystrom et al., 1997; Sandhu and

Gyles, 2002; Stoffregen et al., 2004; Woodward et al., 1999). This work aims to investigate the pathological changes and innate immune responses to *E. coli* O157:H7 colonisation at its main tropism site: the terminal rectum.

4.2 Aims and objectives

1- To review earlier findings that the tropism for the terminal rectum may be related to the presence of lymphoid tissue.

2- To determine by immuno-gold labelling and electron microscopy whether there is type III secretion-mediated mucosal attachment at the terminal rectum.

3- To describe the bacterial adherence to the terminal rectal epithelium by microscopic and ultrastructural examination.

4- To determine if colonisation of the terminal rectal epithelium induced histopathological changes in the terminal rectum.

5- To establish if colonisation of the terminal rectum induces innate mucosal immune responses.

6- To develop a method to sample terminal rectum tissue in infected animals with minimal invasion.

To achieve these objectives the following approaches were taken:

Gastrointestinal tissues from 22 animals from group 1 (section 2.5 M&M) were histologically examined, and additionally 11 animals from the same group had rectal biopsies taken pre-challenge and on two further occasions at days 6 and 12 after challange.

Identification of *E. coli* O157:H7 by indirect immunohistochemistry, immunofluorescence and immunogold labelling.

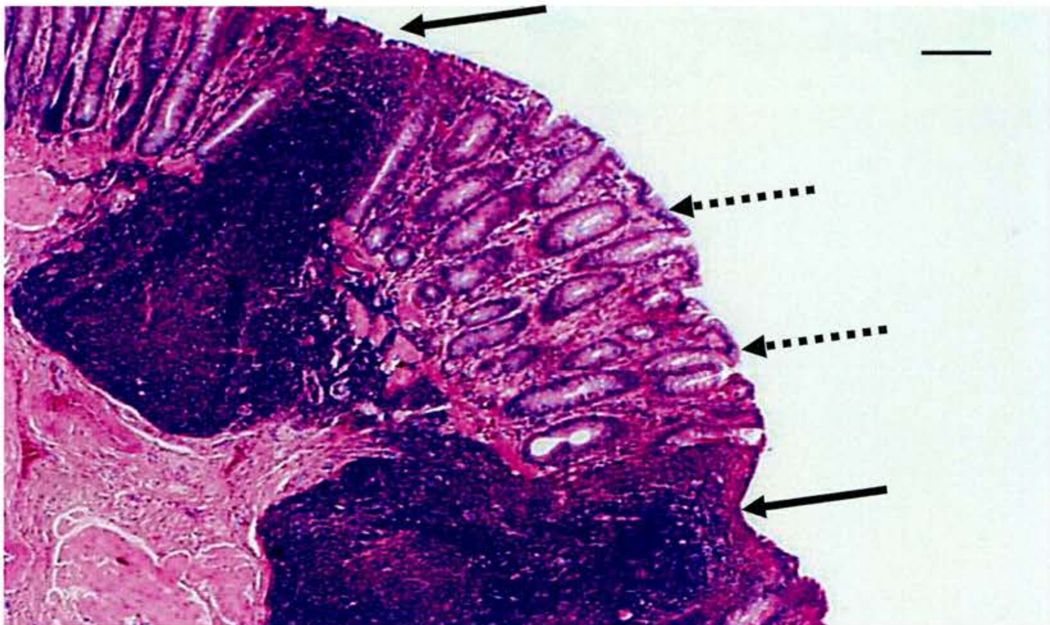
Identification of polymorphonuclear leukocytes and lymphocytes by histochemistry and immunohistochemistry.

4.3 Results

4.3.1 Histological analysis

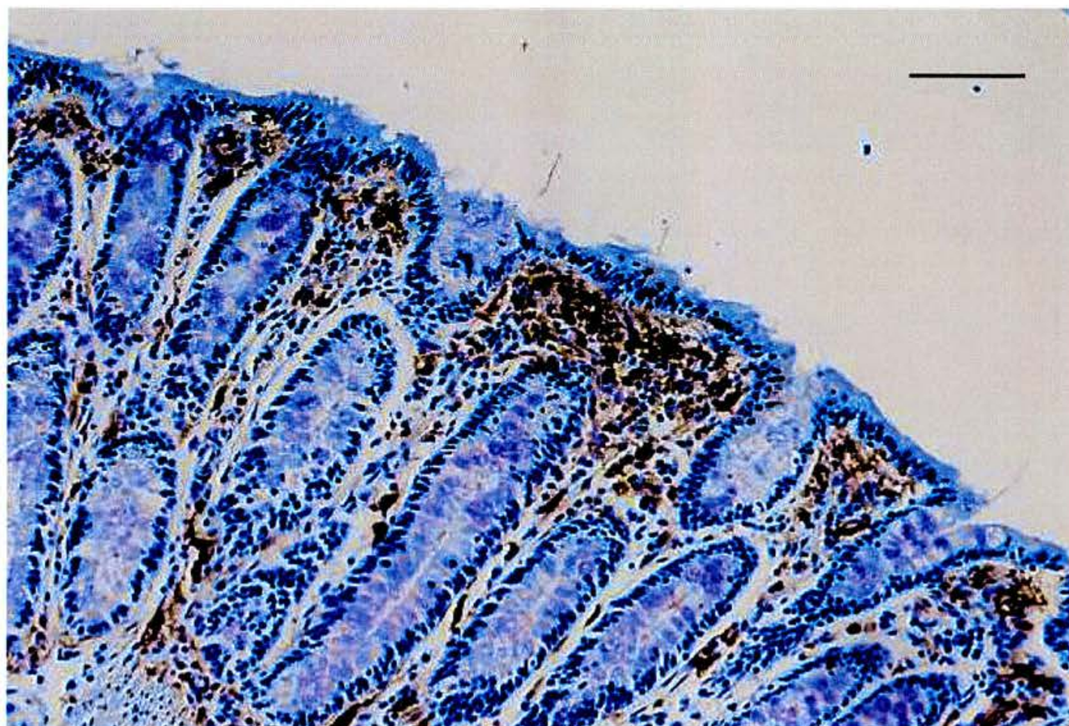
The terminal rectum epithelial cells are phenotypically heterogeneous. Morphologically, examination of Haematoxylin and Eosin (H&E) stained tissues revealed an epithelial cell population composed of low, densely packed, cuboidal follicle-associated epithelium covering lymphoid follicles alternating with simple columnar absorptive epithelium with their nucleus located in the basal third of the cytoplasm (Figure 4-1).

Figure 4-1. Terminal rectum histology H&E, bar = 120 µm. Low cuboidal epithelium lines the lymphoid follicles domes (solid arrows), while simple columnar epithelium rests upon the lamina propria (dotted arrows)



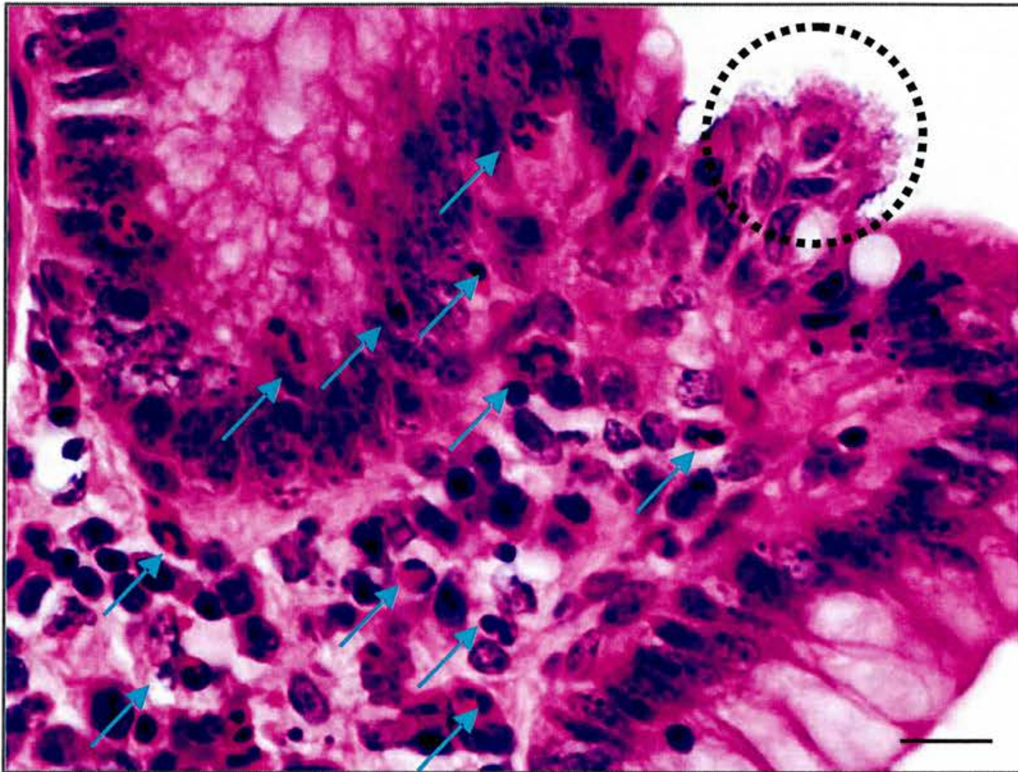
Widespread MHC class II expression was detected by immunohistochemistry on irregular lamina propia cells underlying the intestinal epithelium (Figure 4-2).

Figure 4-2. Terminal rectum. Immunohistochemical staining for MHC class II, bar = 60 μm. Extensive labelling of MHC class II was observed on cell populations subjacent to the gastrointestinal epithelia



Examination of H&E stained tissues failed to detect bacteria or pathological changes in the small intestine or large intestine except at the terminal rectum. Microscopic inspection revealed sparse focal clusters of granulocytes mostly associated with microcolonies of rod-like bacteria, when bacteria at terminal rectal epithelium exceeded 10^5 CFU cm^{-2} (Figure 4-3).

Figure 4-3. Terminal rectum. H&E, bar = 20 µm. A granulocytic infiltrate is present throughout the lamina propia (arrows), Note a bacterial colony on the epithelial luminal surface (dotted circle)



Multifocally, moderate numbers of bacteria are present along the enterocyte brush border and in the intestinal lumen. Occasionally, the epithelium is shedding cells (Figure 4-4). Exuded granulocytes were frequently observed (Figure 4-5).

Figure 4-4. Terminal rectum, H&E, bar = 20 µm. Large numbers of bacteria are noted on areas of sloughed epithelium (dotted circle) accompanied by some granulocytes in the underlying lamina propria (arrows)

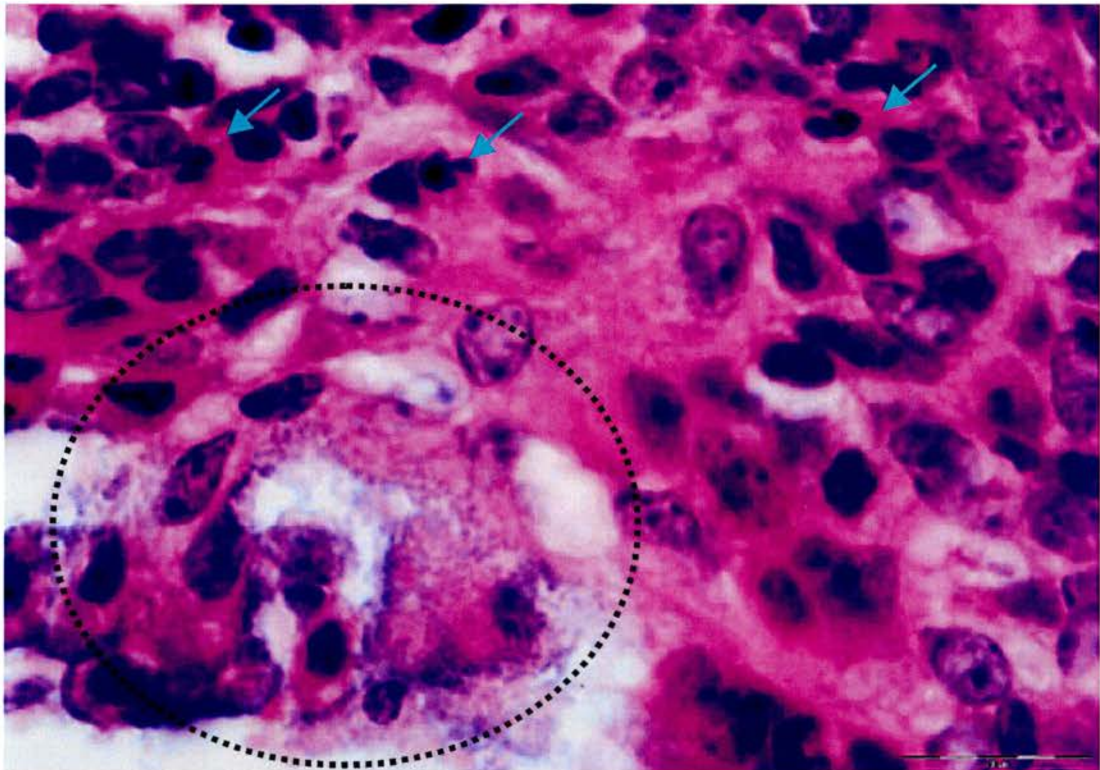
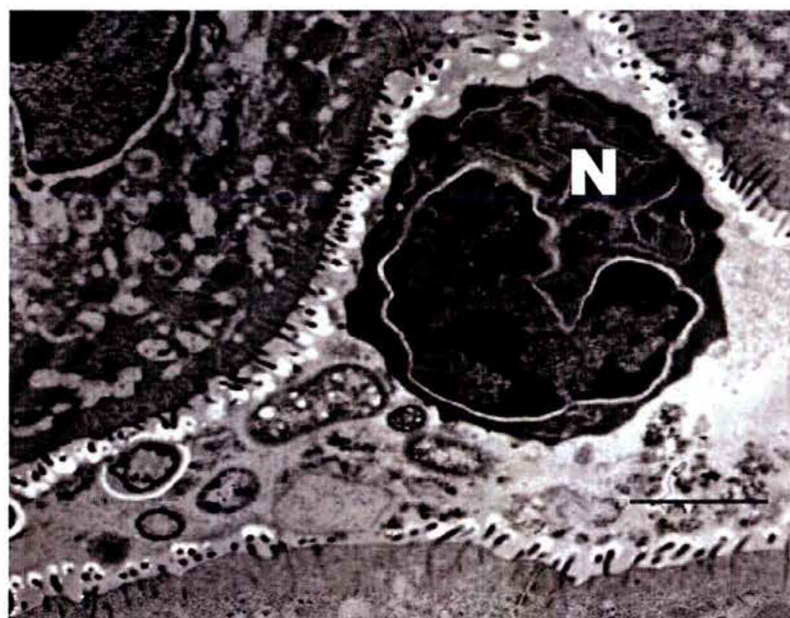


Figure 4-5. Terminal rectum, TEM, bar = 2 µm. Extravasated polymorph mononuclear leukocyte (N).

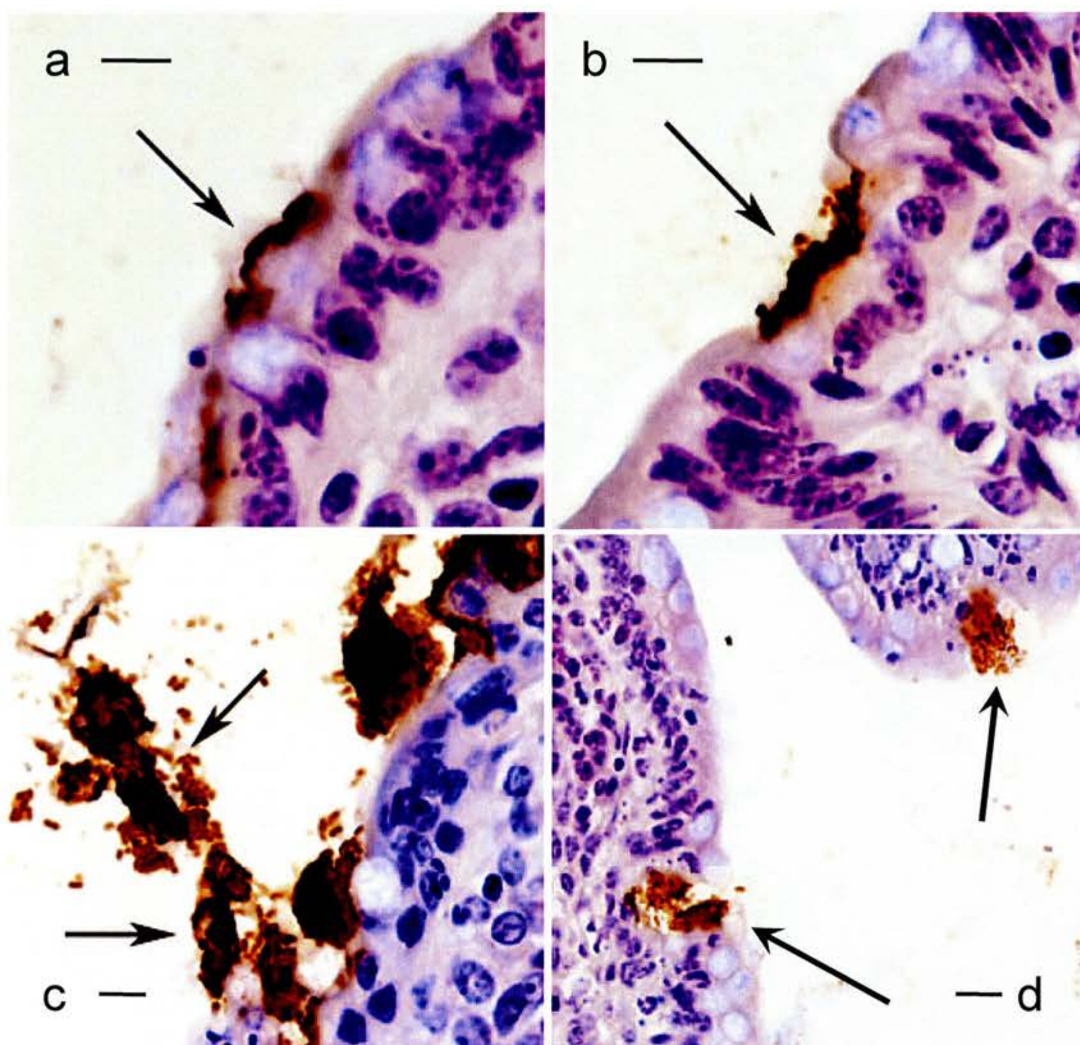


E. coli O157:H7 could be readily detected associated with the epithelium using immuno-staining and microscopy, in animals with bacterial counts over 10^5 CFU g^{-1} (Annex Table IV). In these positive tissues, collected between 14 and 22 days after challenge, the immunopositive bacteria were usually colonising focal areas of the absorptive epithelium or less frequently, the scarcer follicle associated epithelium (FAE).

Bacterial micro-colonies ranged from those containing less than thirty bacteria to those with several hundred. The distribution of the colonies in the terminal rectum appeared random, with some micro-colonies close together, and others separated by large areas of non-colonised rectal tissue. In all cases affected epithelial cells had bacteria intimately associated with their apical membranes. Occasionally, immunostained bacteria were present without producing major morphological alterations to the rectal epithelium.

The mucosal border in foci with attached bacteria was low columnar to cuboidal (Figure 4-6 a and b) with frequent exfoliation of the mucosal epithelium from the basal membrane (Figure 4-6 c) and bacteria often seen in cavities of evacuated enterocytes (Figure 4-6 d).

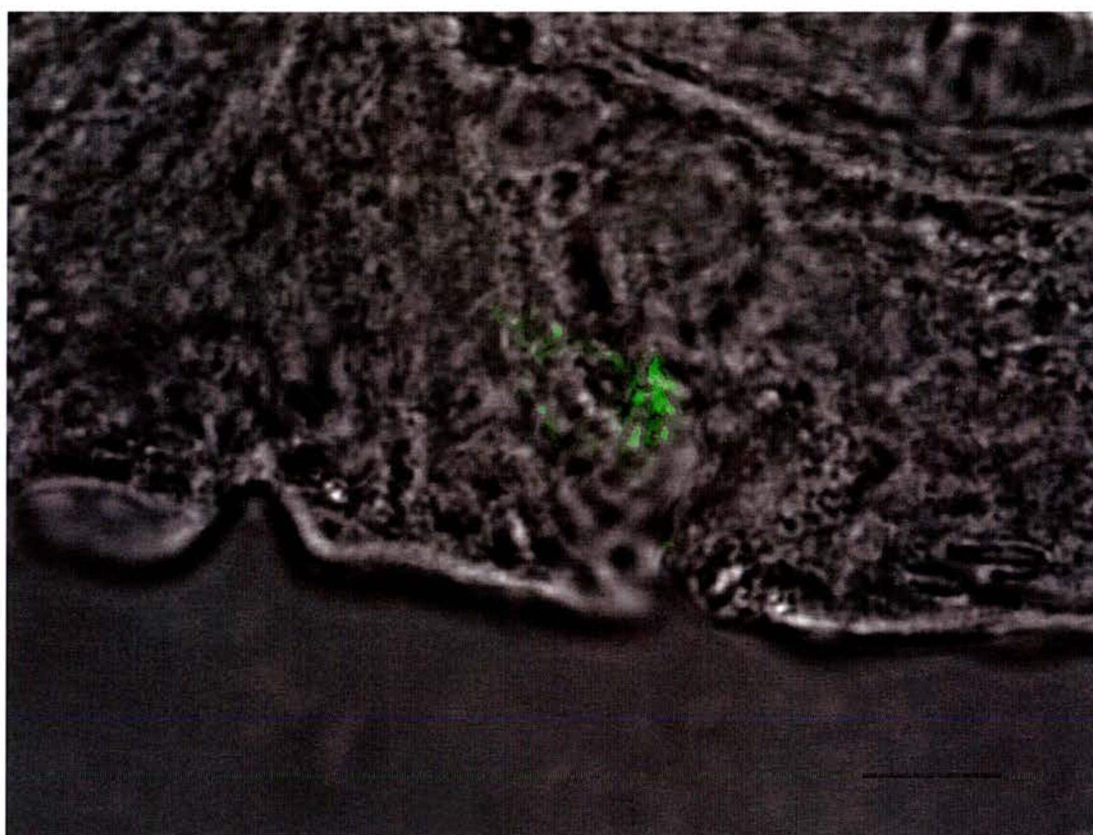
Figure 4-6. Terminal rectum. Immunohistochemical staining for *E. coli* O157:H7 lipopolysaccharide; figures a and b bar = 10 μm; c bar = 20 μm; d, bar = 30 μm. Bacterial colonies (arrows) are seen closely associated with the epithelium (a, b). In some areas heavily colonised enterocytes are sloughing off (c) and bacterial colonies are apparently colonising the cavities of evacuated enterocytes (d)



Groups of loose immunopositve bacteria were also present in the mucus 40 to 100 microns from the luminal surface. On rare occasions *E. coli* O157:H7 was also attached to areas of the squamous epithelium of the perianal region and crypts of the rectal mucosa.

On one occasion, labelling of O157 LPS antigen was found by confocal microscopy within the cytoplasm of a cell of dendritic morphology closely associated with the gastrointestinal epithelium. The abundant immunopositive material was amorphous and lacked the usual rod shape morphology associated with O157 LPS labelling in the other sections examined (Figure 4-7).

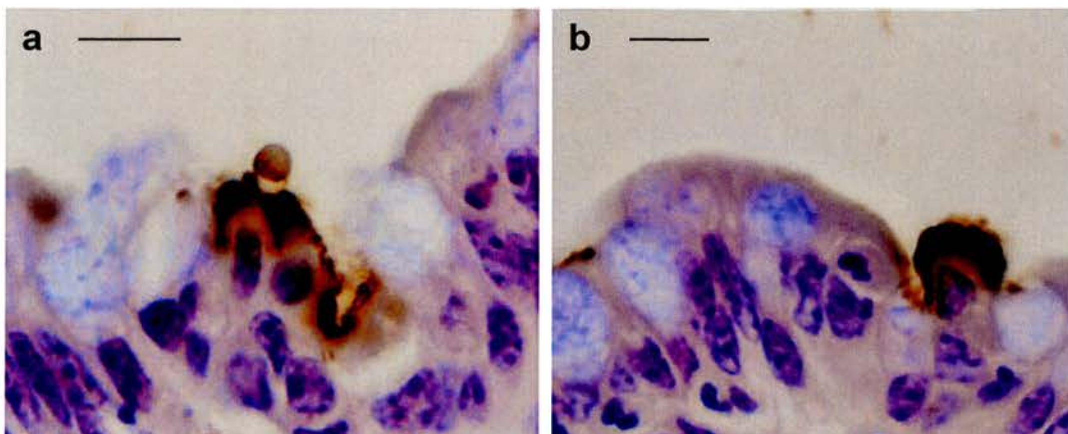
Figure 4-7 Terminal rectum, bar = 2 µm. Confocal micrograph of internalised immunofluorescent amorphous antigen. Immunohistochemical staining for *E. coli* O157:H7 lipopolysaccharide.



Biopsies: *E. coli* O157:H7 micro-colonies were also detected by immunostaining at day 6 from rectal biopsy samples from three animals that were shedding 10^5 CFU g^{-1} *E. coli* O157:H7 in faeces (Figure 4-8, Annex Table V). The distribution and intimate bacterial attachment to

enterocytes was similar to that observed in the cases examined at *post-mortem* on days 15 and 21 post-challenge.

Figure 4-8. Terminal rectum biopsies. Immunohistochemical staining for *E. coli* O157:H7 lipopolysaccharide, figures a and b bar = 10 µm. Immunolabeled bacteria are present closely adherent to the mucosal epithelia.

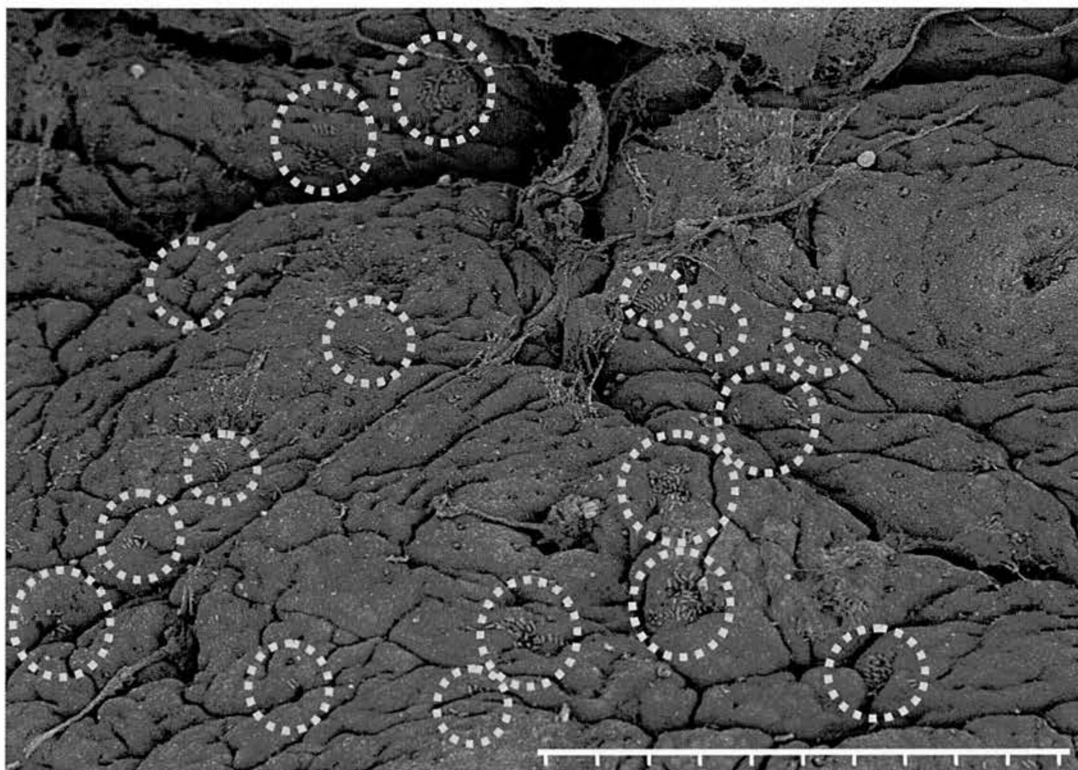


Naturally colonised animal: Histological examination of the terminal rectum tissue from a naturally colonised calf from group 1 (section 2.2 M&M) with over 10^5 CFU cm^{-2} of *E. coli* O157:H7 revealed similar bacterial distribution and histological changes as described above for experimentally colonised animals.

4.3.2 Ultrastructural analysis

Tissues from Group 1 animals examined by immunohistochemistry were further analysed by SEM. Multi-focal clusters of rod-shaped bacteria of up to two microns in length were distributed randomly over the surface of the absorptive epithelium of the rectum (Figure 27).

Figure 4-9 Terminal rectum, SEM. Bacterial microcolonies marked with dotted circle. Bar = 100 µm



Conventional TEM carried out on reprocessed Group II paraffin blocks, and gold particle immuno-labelling TEM carried out on Osmium-free fixative samples from Group 3 animals, allowed identification of *E. coli* O157:H7 on pedestals as part of AE lesions, (Figures 4-10 and 4-11 respectively) and attaching intimately to the enterocyte apical surface (Figure 4-12). All the electron micrographs were taken from animals orally challenged, except figure 4-12 that was taken from an animal challenged *per-rectum*.

Figure 4-10. Terminal rectum, TEM. Section through an *E. coli* O157:H7 micro-colony at the bovine terminal rectum. The bacteria are all intimately attached (black arrows) to the damaged epithelium, inducing effacement of the microvilli. Unaffected brush border is visible on the neighbouring epithelia (white arrow), bar = 1 μm.

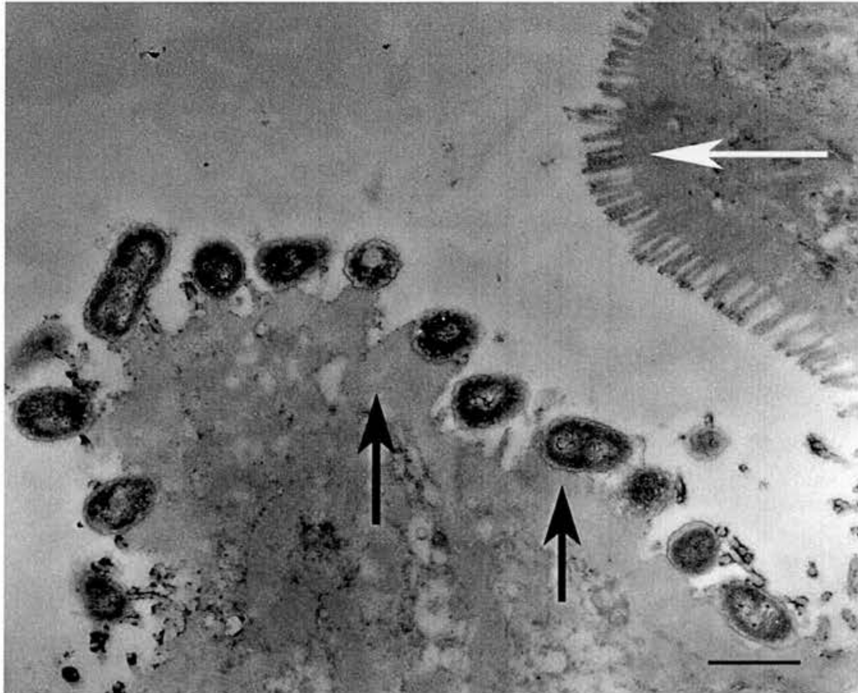


Figure 4-11 Terminal rectum, TEM. Immunogold labelling of E. coli O157:H7, bar = 250 nm, arrows point to gold particles.

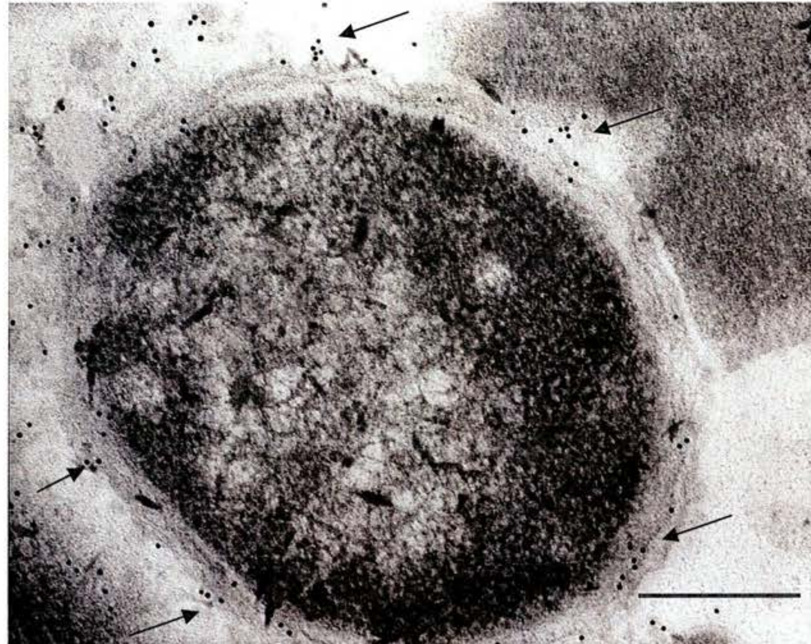
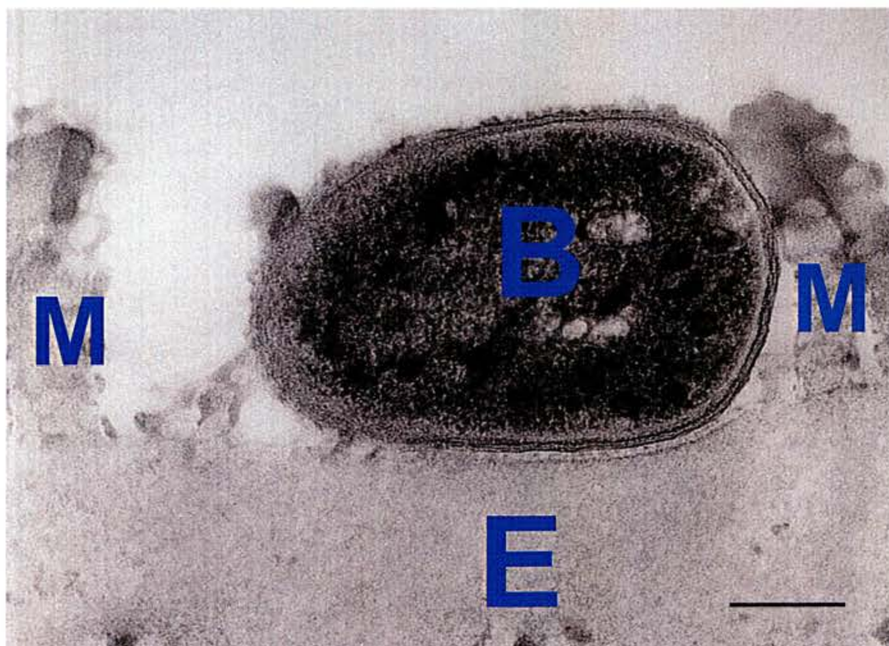
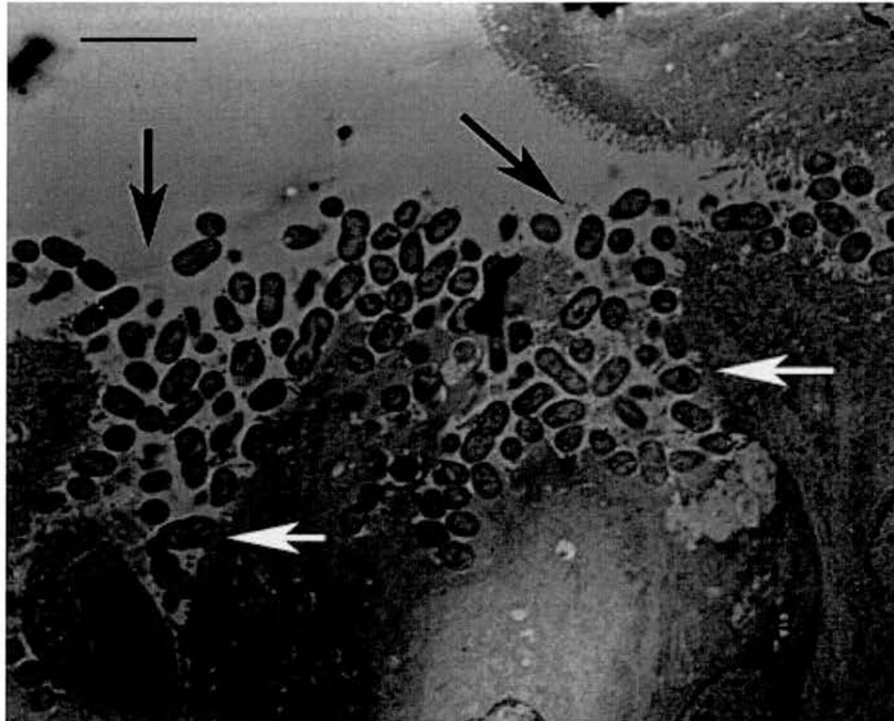


Figure 4-12. Terminal rectum, TEM. Bacterium (B) intimately attached to the apical membrane of an enterocyte (E) surrounded by microvilli (M), bar = 500 nm.



Pedestal heights varied but in some cases were up to 10 microns. Some micro-colonies consisted of bacteria in layers, apparently forming a stack and individual bacteria were observed in the process of dividing. Bacterial micro-colonies were associated with different degrees of enterocyte erosion (Figure 4-13).

Figure 4-13. Terminal rectum, TEM. Aggregates of bacteria accumulate over the roughened surface of the colonised enterocytes. Note the severe cytoplasmic alterations in the form of deep pits (black arrows). Bacteria are intimately attached to the apical surface of the enterocyte (white arrows), bar = 5 μm.



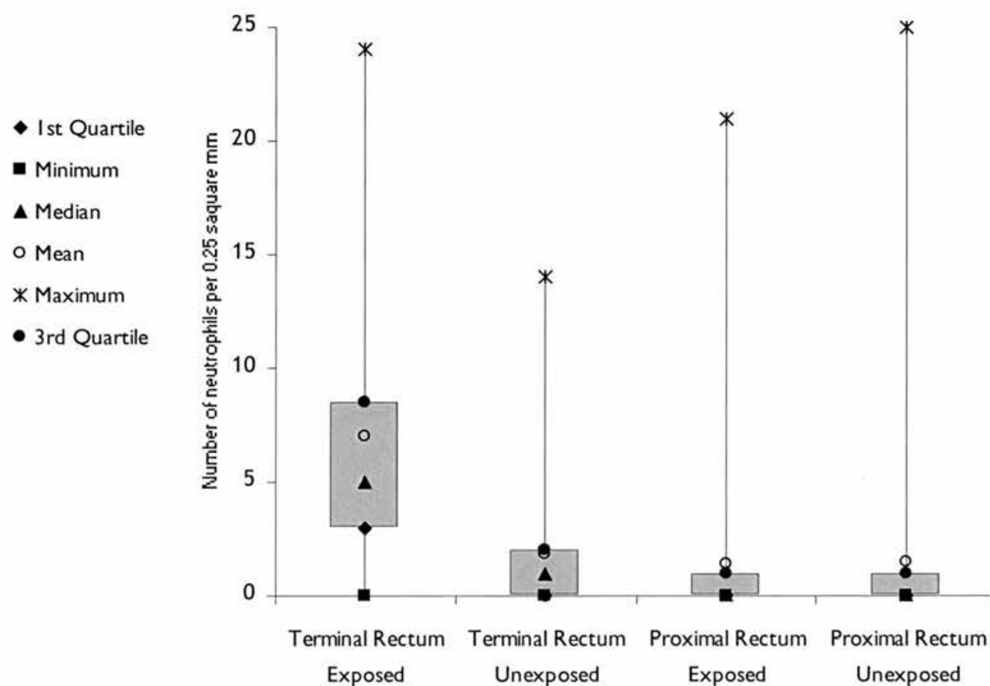
4.3.3 Granulocytic infiltration

Microscopic examination of biopsy and *post-mortem* rectal tissue colonised with *E. coli* O157 demonstrated a diffuse, low to mild granulocytic focal infiltration of the lamina propria. On occasions granulocytes were present interspersed amongst enterocytes and exuded leucocytes formed aggregations in the gut lumen (micro-abscesses).

In the terminal rectum, a significant ($P<0.001$) granulocytic infiltrate was present in colonised animals (mean 7.0 ± 1.4 per 0.25 mm^2) when compared with controls (mean 1.9 ± 0.9 per 0.25 mm^2). For colonised animals significantly more granulocytes ($P<0.001$) were found in the terminal rectum compared to the proximal rectum (+20cm). In the area twenty centimetres proximal to the terminal rectum, no significant differences ($p>0.42$) in granulocytes counts were detected between infected and non-infected cases (controls mean 1.2 ± 1.1 per 0.25 mm^2, cases 1.5 ± 1.0 per 0.25 mm^2) (Figure 4-14, Annex Table VI).

Figure 4-14. Box plot representation of neutrophils numbers obtained in the terminal and distal rectum of exposed and unexposed animals

The boxes contain 50% of the data and the median count is illustrated by the black triangle. Samples were prepared and granulocytes identified as described in section 2.13, M&M.



However, no differences were detected between numbers of eosinophils, mast cells and $\gamma\delta$ T-cells (P>0.65; P>0.69 and P>0.68) when compared in colonised and control animals.

4.4 Discussion

E. coli O157:H7 causes pathological changes to the mucosal epithelium of the rectum and the changes are histologically detectable in the rectum when numbers are over 10^5 CFU cm^{-1} of *E. coli* O157:H7 on tissues. The main histopathological findings at sites of bacterial attachment were: (i) attaching and effacing lesions, (ii) in heavily colonised cells a reduction of enterocyte cytoplasmatic height and degeneration, and (iii) frequent enterocyte sloughing. The microscopic examination was made in animals shedding bacterial numbers similar to those animals considered high shedders in field studies (Stanford et al., 2005; Fegan et al., 2004) and importantly, similar changes were detected in the terminal rectum of a naturally infected animal. No alterations in the integrity of the epithelial mucosa were observed in control animals or in colonised animals shedding below the 10^5 CFU g^{-1} threshold

In cell culture, *E. coli* O157:H7 strains induce apoptosis in human epithelial (HEp-2) cells resulting in increased surface-exposed phosphatidylethanolamine levels and enhanced attachment (Barnett Foster et al., 2000). In our study, examination by electron and light microscopy of considerable areas of the rectal mucosa with varied levels of colonisation, failed to detect the, certainly rapid event, of enterocyte apoptosis. Given the severe nature of the enterocyte cytoplasmatic changes observed during the different phases of bacterial colonisation, it is possible that most of the mucosal damage inflicted by *E. coli* O157:H7 may be due to enterocyte desquamation. *In vitro* studies have consistently reported a decreased transepithelial resistance and opening

of the tight junctions following *E. coli* O157:H7 colonisation of monolayers (Philpott et al., 1998; Tomson et al., 2004).

We have confirmed that *E. coli* O157:H7 persist in the bovine host forming AE lesions in animals with bacterial counts over 10^5 CFU g^{-1} of *E. coli* O157:H7 in terminal rectum tissues collected two and three weeks after experimental inoculation, allowing the bacteria to establish a long-term persistence, similar in duration to the natural carriage observed in animals in field studies (Besser et al., 1997). Similar lesions have been previously reported in weaned calves four days post-challenge (Dean-Nystrom et al., 1999), and bacteria closely associated to the terminal rectum has also been reported in weaned calves (Naylor et al., 2003b). In addition, in this work we present ultrastructural evidence of AE lesions at the terminal rectum and specific immunogold labelling of *E. coli* O157:H7 lipopolysascharide. This methods are considered definitive for establishing the presence of AE lesions (Wales et al., 2001a, Wales et al., 2005a).

The destructive alterations induced by *E. coli* O157:H7 observed in the bovine epithelial barrier induced a quantifiable neutrophilic response in cattle. This type of inflammatory reaction has been previously described in the intestinal tract for experimental infections of gnotobiotic or neonatal calves (0 to 1 day old) that are not completely immunocompetent. A quantified increase in neutrophils and eosinophils in the lamina propria has been described in one occasion (Woodward et al. 1999), while other studies report similar increases of leucocytes in colon and caecum (Dean-Nystrom et al., 1997), sections of ligated ileal

loops (Sandhu and Gyles, 2002) and perianal skin (Pohlenz and Dean-Nystrom, 2004). This is the first report of neutrophilic responses at the terminal rectum to *E. coli* O157:H7 rectal colonisation in weaned calves.

In vitro adherence studies made with *E. coli* O157: H7 in intestinal organ cultures of human small and large intestine epithelium showed that attachment was mediated by intimin gamma and restricted to follicle associated epithelia of Peyer's patches (Phillips et al., 2000). The terminal rectum is an area rich in lymphoid follicles (Mahajan et al., 2005) and previous work has suggested a similar association to explain *E. coli* O157:H7 tropism for the bovine terminal rectum (Naylor et al., 2003a). In this study, extensive histological examination of terminal rectum tissues did not reveal a prominent association between *E. coli* O157:H7 microcolonies and follicle associated epithelium. Although the principal site of *E. coli* O157:H7 colinization is the lymphoid follicle-dense mucosa of the terminal rectum, *E. coli* O157:H7 attachment to the terminal rectum was not restricted to any particular epithelia cell type (follicle associated epithelium, simple columnar epithelium or goblet cells) present in the area.

Enteropathogenic *E. coli* and non-O157:H7 enterohaemorrhagic strains have a wide ranging tropism for the gastrointestinal tract and AE lesions have been described in a wide range of species species including rabbits (Cantey and Blake, 1977); dogs (Drolet et al., 1994a); goat (Drolet et al., 1994b); calves (Hall et al., 1985); lambs (Janke et al., 1989);, cats (Pospischil et al., 1987) pigs (Staley et al., 1969) and in the large intestine and rectum of sheep (Wales et al., 2005b). The reason for the terminal

rectum tropism of *E. coli* O157:H7 in cattle is still obscure. Characteristically, *E. coli* O157:H7 expression of fimbrial adhesins is precluded by gene mutations in all but three type IV fimbrial clusters, and subsequent studies have concluded that these adhesins are not involved in cattle colonisation (Low et al., 2006a), limiting the putative adherence factors to other molecules of the cell wall. A possible explanation for terminal rectum tropism may be related to friction and the particular anatomy of the area. Two of the main features are the reduced width of the mucous barrier over the Peyer's patches that populate this particular area and that the terminal rectum junction is subjacent to the anal sphincter (Sansonetti, 2004). This combined effect of a reduced protective mucin barrier coupled with raised intrarectal pressure during defecation, may facilitate the colonisation of the area, by promoting cell to cell contact, one of the key mechanisms that induces the expression of the type III secretion system (Roe et al., 2003).

The formation of attaching and effacing (AE) lesions, characterised by elimination of the microvilli and intimate enterocyte attachment (Dean-Nystrom et al. 1999; Ismaili et al. 1998, Moon et al., 1983) produces a substantial damage to the host epithelial cell and is unlikely to be unnoticed by the host adaptative immune system. This work provides evidence for epithelial and inflammatory changes associated with colonisation of the terminal rectum, similar to reported changes at other sites in the intestinal tract (Woodward et al., 1999; Dean-Nystrom et al., 1999; Wales et al., 2001), and inflammatory changes in response to *E. coli* O157: H7 carriage. These inflammatory responses are mild and affect a limited area of the rectum. However, the changes may be indicators of

the possible development of acquire immune responses to *E. coli* O157: H7 colonisation that may offer a means to control the carriage of this important human pathogen and the development of acquired mucosal immune response will now be investigated.

Chapter 5

Mucosal antibody responses in the terminal rectum of *E. coli* O157:H7 colonised cattle

5.1 Introduction

The presence of tissue damage and inflammation caused by pathogens, is generally followed by a vigorous adaptive immune response (Hamburger et al., 2006). The mucosal immune system is equipped with secretory immunoglobulin type A, an acquired defence mechanism that provides a first line of protection against enteric pathogens (Fagarasan, 2006).

Humoral immunity in mucosal secretions is mainly associated with polymeric immunoglobulin A (IgA) bound to the secretory component as secretory IgA (S-IgA). IgA is secreted into the intestinal lumen by an IgA-specific Fc receptor called poly-IgG (pIg) and are more resistant to digestion by microbial and intestinal proteolytic enzymes than other isotypes (Brandtzaeg, 2003). IgA produced by B cells in the lamina propria is secreted as dimers or polymers bound to the joint (J) chain a characteristic protein component of mucosal secreted antibodies. The pIg receptor has an associated extracellular molecule secretory component (SC) that is responsible for binding and transport through the enterocyte of poly-IgA and with less efficiency to poly-IgM than to poly-IgA. The SC-Ig complex is found bound to secreted IgA (sIgA) and IgM. The bovine receptor has been well characterised biochemically (Butler, 1986). Certain species such as cattle and horses also have equal amounts of IgG in the intestinal fluid which is usually considered serum derived (Snoeck et al., 2006; Widders et al. 1984). Importantly for *E. coli* O157:H7 colonisation, IgA is the predominant isotype at the rectum with a ratio of 5:1 (McNeilly et al., 2007).

Serological and mucosal immune responses have been reported previously in cattle. Serum IgG-associated responses have been reported against a variety of antigens, (Cray Jr. and Moon, 1995; Johnson et al., 1996; Laegreid et al., 1999; Pirro et al., 1995; Potter et al., 2004; Wray et al., 2000) while mucosal IgA-associated responses have been reported against *E. coli* O157:H7 intimin (Yokomizo et al., 2002) and flagellin (McNeilly et al., 2007). Nevertheless, no attempt has been made to characterise the gastrointestinal mucosal humoral response to *E. coli* O157:H7 infection, in particular at the principal site of colonisation at the terminal rectum.

5.2 Aims and objectives

1- To investigate the presence of local mucosal antibody responses.

2- To define those antigens recognised during colonisation.

3- To determine rectal mucosal levels of IgA and IgG following infection.

To achieve these objectives the following approaches were taken:

To establish a reliable method of extraction of mucosal antibody.

To establish a 1-D SDS-PAGE and immunoblotting system for whole *E. coli* O157 and cell fraction preparations (sections 2.19 and 2.21, M&M).

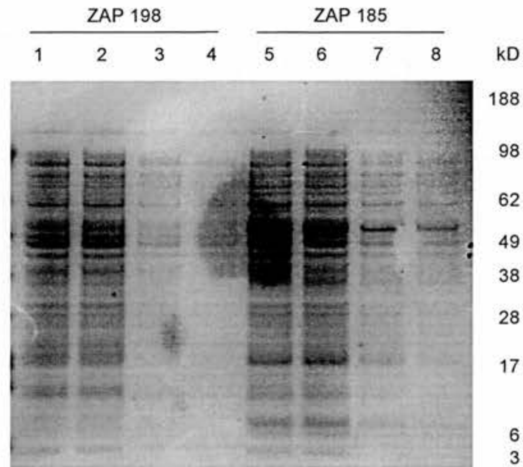
To quantify by ELISA test the immune responses to two main *E. coli* O157:H7 antigens, namely O157 LPS and H7 flagellin (section 2.17 M&M).

5.3 Results

5.3.1 Immunoblotting

To examine whether animals colonised by *E. coli* O157:H7 at the terminal rectum were generating specific mucosal antibody responses to *E. coli* O157:H7 antigens, samples were taken initially from three animals from Group 1 (204, 722 and 868) that shed *E. coli* O157:H7 at levels consistently over 10^4 CFU $gram^{-1}$ for at least one week, as it was thought that the concentration of specific antibodies would be higher in these animals. Mucosal antibodies were extracted from rectal mucosal homogenates as described in section 2.6 of M&M and used for immunoblotting. The experiments were carried out using as antigens whole cell samples from the challenge strain *E. coli* O157:H7 (ZAP 198) and a negative control laboratory strain *E. coli* K12 MG1655 (ZAP 185). Initially the antigen was titrated by reducing 1-D SDS-PAGE gel electrophoresis and the proteins visualised by Simply Blue stain (Figure 5-1) as described in section 2.19 of M&M.

Figure 5-1. Reducing 1-D SDS/PAGE analysis of whole cell samples of *E. coli* ZAP 198 and *E. coli* ZAP 185. Simply Blue stained proteins

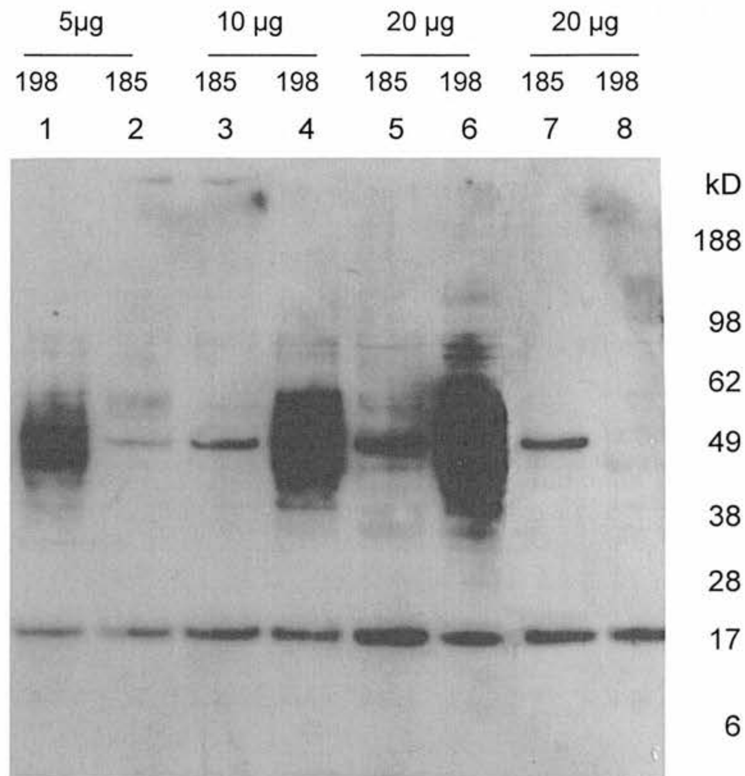


Lanes 1-4 loaded with whole cell samples of ZAP 198 (1) 40µg, (2) 20µg, (3) 10µg, (4) 5µg

Lanes 5-8 loaded with whole cell samples of ZAP 185 (1) 40µg, (2) 20µg, (3) 10µg, (4) 5µg

It was considered that Simply Blue stain of proteins was likely to be less sensitive than Western blotting and chemiluminescence detection of protein bands. To minimise non-specific reactions the lowest antigenic loads, which provided distinct visible bands, were used in subsequent Western blotting experiments. Rectal tissue extracts of case 204 (Figure 5-2) were used to incubate the transfers at a tentative 1:150 dilution.

Figure 5-2. Reducing 1-D SDS/PAGE Western blot analysis of serial concentrations of whole cell samples *E. coli* ZAP 198 and *E. coli* ZAP 185 incubated with rectal mucosal extract form case 204. Dilution 1:150, exposure 10 s bioting conjugated tertiary antibody.



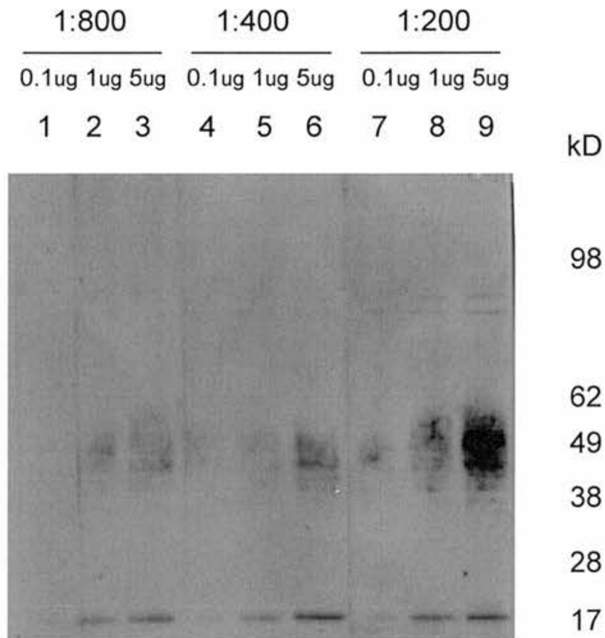
ZAP 198, lanes 1 (5µg), 4 (10µg), 6 (20µg) and 8 (20µg).
ZAP 185, lanes 2 (5µg), 3 (10µg), 5 (20µg) and 7 (20µg).

In this blot, IgA reactivity against ZAP 198 was demonstrated as a pronounced but indistinct area at approximately 38-98 kD, with an additional strong band at 16 kD. In contrast, the reactivity to ZAP 185 was markedly less, showing two bands at 49 kD and at 16 kD. The total protein antigen load chosen for further immunoblotting was 5µg.

To establish the working dilution of the extracts of rectal mucosa and to test lower dilutions of antigen, a 1-D SDS/PAGE gel was loaded with

serial dilutions of ZAP 198 antigen. The membrane transfer was then incubated with serial dilutions of extracts from case 722. The combination of 5 µg of antigen and extract dilution of 1:200 was selected for further immunoblot detections (Figure 5-3).

Figure 5-3. Reducing 1-D SDS/PAGE Western blot analysis of whole cell samples *E. coli* ZAP 198 incubated with serial dilutions of of rectal mucosal extracts from case 722. Exposure 10 s bioting conjugated tertiary antibody

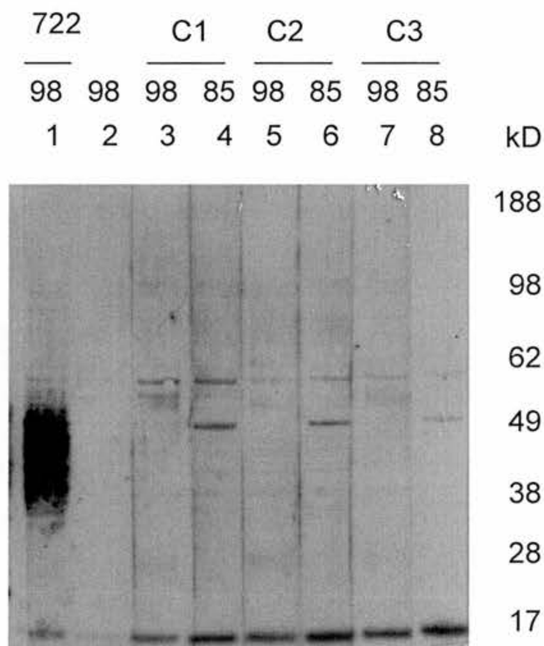


ZAP 198. Lanes 1, 4, 7 (0.1 µg) 2, 5, 8 (1µg) 3, 6, 9 (5 µg)
Incubation transfers 1, 2 and 3 1:800; 4, 5 and 6 1:400; 7, 8 and 9 1:200

Rectal mucosal extracts from case 868 were incubated with transfers of strain ZAP 198 with similar results as shown for case 204 and 722 (result not shown). Negative controls were carried out incubating without pooled rectal extract.

To evaluate the specificity of the immune reaction 1-D SDS-PAGE Western blots were carried out with rectal mucosal extracts from a group of unexposed calves (sections 2.2 and 2.4 M&M). No specific reactive bands were observed (Figure 5-4).

Figure 5-4. Reducing 1-D SDS/PAGE Western blot analysis of whole cell samples *E. coli* ZAP 198 incubated with rectal mucosal extracts from three animals from control group 5. Exposure 10 s bioting conjugated tertiary antibody.



ZAP 198 (98), lanes 1,2 3, 5, 7; ZAP 185 (85) lanes 4, 6, 8

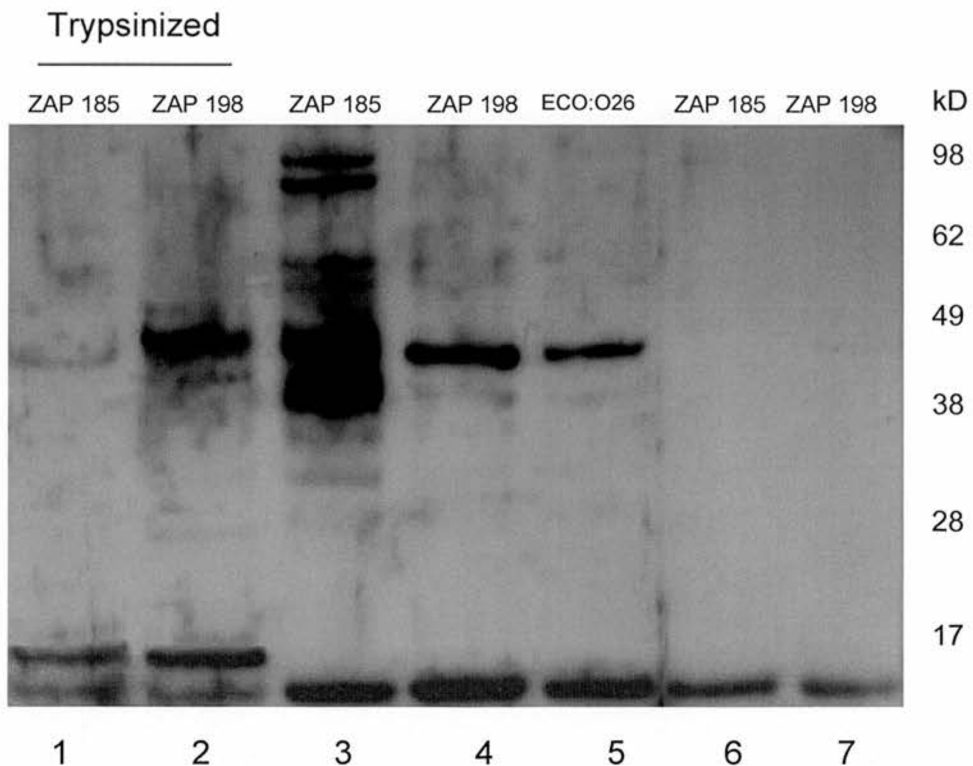
Strip 1 positive control ZAP 198 incubated with case 722, extract dilution 1:200. Strip 2 negative control no extract.

Strips 3-4 incubated with control 1 (C1), strips 5-6 control 2 (C2) and strips 7-8 control 3 (C3)

To further verify the specificity of the immune reaction and to asses the contribution of the protein antigen to the immunoreactivity, extracts of rectal mucosa were used to blot whole cell preparations of *E. coli* strains

O157 ZAP 198, O26 and K12 ZAP 185. It can be seen that multiple immunoreactive bands were detected from the *E. coli* O157 strain by comparison with *E. coli* O26 and K12. Trypsin digestion of the samples removed most of the immunoreactive material, indicating the immunodominance of the protein component in the blotted antigen. Lanes six and seven are controls containing *E. coli* O157 and K12 antigens incubated with all the reagents except the rectal homogenate in order to detect non specific reactions (Figure 5-5).

Figure 5-5. Reducing 1-D SDS/PAGE Western blot analysis of whole cell samples *E. coli* ZAP 198 incubated with rectal mucosal extracts from case 868, lanes 6 and 7 no extract. Exposure 10 s bioting conjugated tertiary antibody.



Lanes (1) trypsinised ZAP 185, (2) trypsinised ZAP 198, (3) untreated ZAP 198, (4) untreated ZAP 185, (5) untreated *E. coli* O26, (6) ZAP 185 (7) ZAP 198

Having detected specific immune responses against whole cell antigens of ZAP 198 in three animals with visible histological lesions at the rectum in *post-mortem* samples, it was decided to extend the work and challenge a second group of eight animals (Group 2, section 2.2 M&M). This second group would enable us to sample animals pre-initial challenge to obtain mucosal rectal samples by rectal biopsy, a technique that was developed during the initial histological studies (section 2.4 M&M).

During this study, faeces were sampled 38 times from each animal during a 50 day period after initial challenge. The counts obtained for each animal are shown in Table 5-1. All animals had faecal samples with detectable levels of *E. coli* O157:H7 from day 6 and until day 21, with average levels above 10^2 CFU g^{-1}. After rechallenges on day 35 and 36 animals shed similar levels of bacteria until day 40. Maximum levels detected in individual animals ranged from 1.3×10^3 to 2.4×10^5 CFU g^{-1} of *E. coli* O157:H7. The average bacterial count for all the samples collected was 3.5×10^3 CFU g^{-1} of *E. coli* O157:H7. None of the calves reached the continuous levels of shedding above 10^4 CFU g^{-1} of *E. coli* O157:H7 achieved by the animals with histological lesions from Group 1, and therefore no histology was performed. Calves did not show any clinical signs during this experiment. The following table shows the timetable of challenges and sampling and the numbers of bacteria found on the surface of the faeces during the period of study.

Table 5-1. Group 2 calves, challenging / sampling procedures and daily concentrations (CFU g^{-1}) of *E. coli* O157:H7 ZAP 198 detected on the surface of faeces

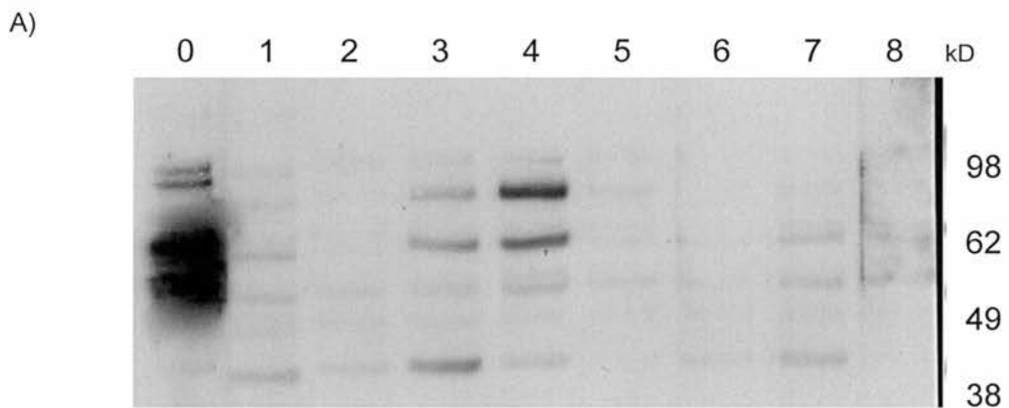
Challenges / Sampling			DPC[1]	Calves							
CH[2]	BS[3]	RB[4]		1	2	3	4	5	6	7	8
X	X	X	0								
			1	5,433	3,133	3,033	233	667	67	5,367	67
			2	0	200	0	67	33	467	0	100
			3	NC[5]	2,000	0	67	0	133	0	400
			4	0	567	0	167	67	600	0	1,300
			5	0	8,967	0	33	67	133	0	667
X		X	6	0	400	0	100	233	533	0	100
			8	933	19,433	100	NC	3,533	700	33	0
			9	500	2,500	67	0	133	7,467	0	0
			10	0	5,033	1,267	267	5,467	21,700	67	0
			11	100	0	21,233	67	NC	3,500	9,733	0
			12	0	NC	NC	200	NC	9,667	2,400	0
			13	0	21,933	63,367	0	56,933	NC	73,133	0
	X		14	0	33	10,700	167	NC	8,233	24,900	0
			15	0	0	37,733	467	267	NC	8,367	0
			16	0	667	50,133	33	11,100	433	1,533	0
			17	0	NC	240,667	133	3,267	1,000	16,867	NC
			18	0	67	51,433	133	67	0	333	NC
			19	0	133	36,600	33	500	167	1,067	0
			20	0	300	267	33	100	100	600	0
			21	0	100	2,533	0	0	33	600	0
X	X	X	35	0	0	0	0	0	0	0	NC
X			36	533	100	2,600	3,300	3,400	2,267	2,300	167
			37	167	0	8,333	3,200	1,900	0	1,467	233
			38	967	33	867	500	NC	133	1,500	0
			39	100	1,467	0	633	14,633	33	1,433	0
			40	3,367	33	33	733	200	0	6,567	433
			41	3,067	33	NC	467	3,633	0	533	0
			42	7,700	0	0	1,133	233	0	7,933	0
			43	2,333	133	0	0	0	0	1,200	0
			44	33	NC	0	0	200	800	767	400
			45		NC	0	67	100	0	100	33
			46	33	67	0	0	0	0	0	100
			47	0	0	0	0	0	0	0	0
			48	67	0	0	0	0	0	133	67
	X		49	0	167	0	0	33	0	0	0
		X	50	0	0	0	0	0	0	0	0

(1) DPC: Days 1 to 50 post initial challenge
(2) CH: *E. coli* O157:H7 ZAP 198 oral challenge
(3) BS: Blood sample
(4) RB: Rectal mucosa biopsy
(5) NC: Sample not collected

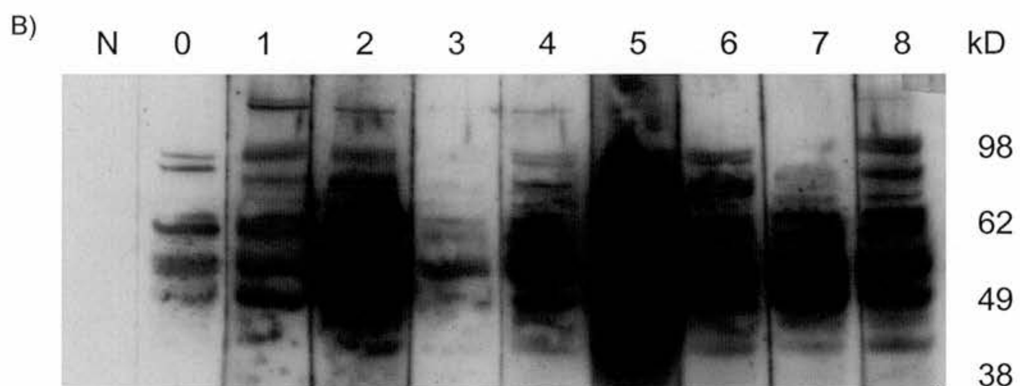
Using mucosal extracts taken at *post-mortem* at day 50 post-challenge IgA antibodies were detected that bound to antigens of whole *E. coli*

O157:H7. Between 4 to 11 protein bands, with molecular weights ranging from 38 and 98 kD were recognised. A fainter and less numerous pattern of reactive bands was observed in pre initial challenge samples from homogenates of rectal tissues obtained by rectal biopsy, as presented in Figure 5-6.

Figure 5-6. Reducing 1-D SDS-PAGE Western blot of *E. coli* ZAP 198 whole cell samples incubated with rectal mucosa extracts obtained from group 2 animals pre-initial challenge (A) and at *post-mortem* (B)



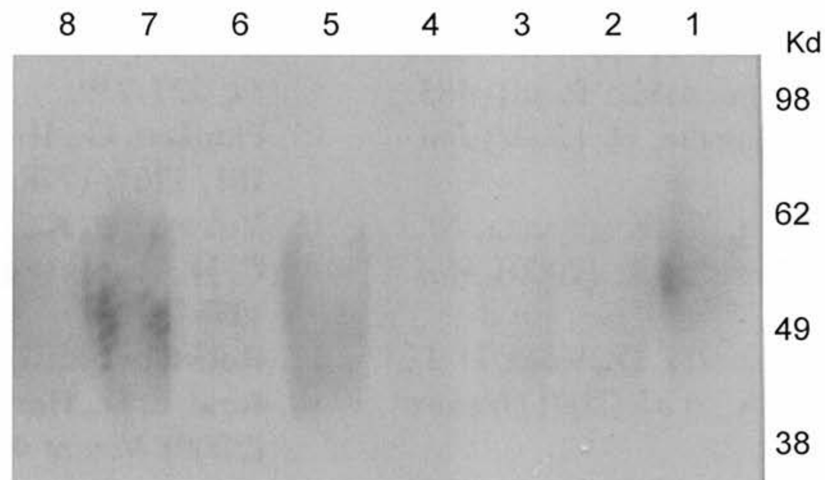
Transfer 0, pooled rectal extract from group 1 cases; transfers 1 to 8, group 2 cases. Exposure time 10 s.



Transfer N, no extract; 0, pooled rectal extract from group 1 cases; transfers 1 to 8, group 2 cases. Exposure time 10 s

To elucidate the immunological relevance of the protein antigens compared to *E. coli* O157:H7 lipopolysaccharide detected by Western blot, the whole cell antigen samples were submitted to enzymatic digestion. Proteinase K digestion of the ZAP 198 (section 2.15 M&M) antigen preparation removed most protein bands detected by IgA, leaving a diffuse area of recognition at 71-55 kD for three cases (Figure 5-7).

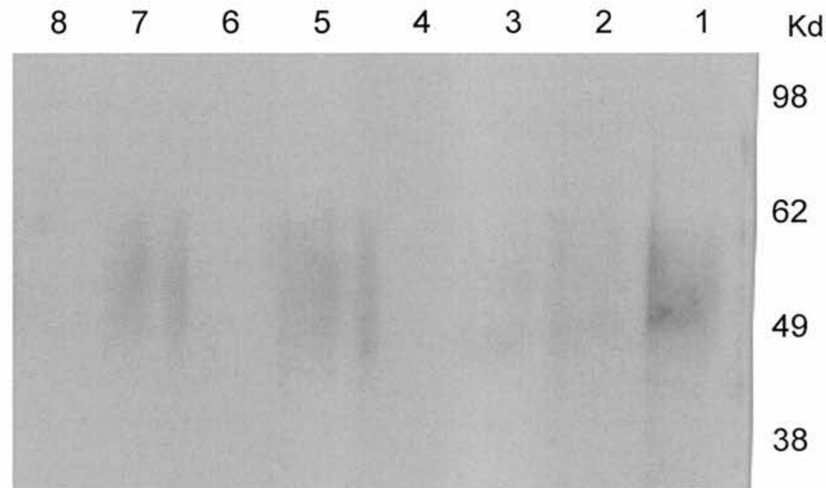
Figure 5-7. Reducing 1-D SDS-PAGE Western blot of proteinase K treated whole cell samples of *E. coli* ZAP 198 samples incubated with rectal mucosa extracts obtained at *post-mortem* from group 2 animals



Transfers cases 1 to 8 group 2. Exposure time 10 s.
All lanes loaded with proteinase K treated ZAP 198.

Furthermore, most of the homogenate samples did not show reactivity to LPS of *E. coli* O157:H7, which appeared as a single diffuse band migrating below the 70-kD marker (Figure 5-8).

Figure 5-8. Reducing 1-D SDS-PAGE Western blot of *E. coli* O157 LPS incubated with rectal mucosa extracts obtained at *post-mortem* from group 2 animals



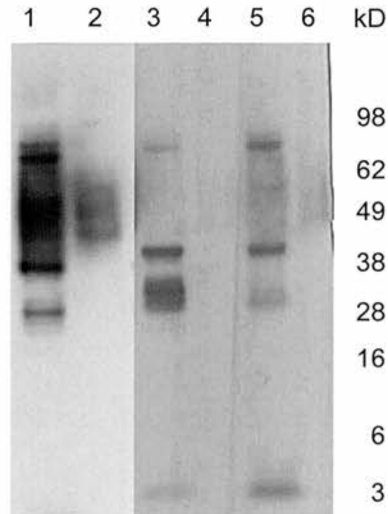
Transfers cases 1 to 8 group 2

Exposure time 10 s. All lanes loaded with O157 LPS.

After establishing the predominance of the protein component in the overall immunoreactivity of the sample, a comparative study of the different secreted and somatic protein fractions of the bacteria was carry out.

Membrane and cytoplasm protein fractions were analysed by 1-D SDS-PAGE immunoblotting. IgA antibody reactivity was predominantly confined to the membrane fractions, although faint and indistinct reaction against cytoplasmic proteins was observed from the pooled material of highly colonised animals. The reactive membrane proteins ranged from 30-90 kD, with three major bands showing at approximately 30, 40 and 70 kD (Figure 5-9).

Figure 5-9. Reducing 1-D SDS-PAGE Western blot of *E. coli* ZAP 198 membrane and cytoplasmatic fractions incubated with rectal mucosa extracts obtained at *post-mortem* from group 1 and 2 animals



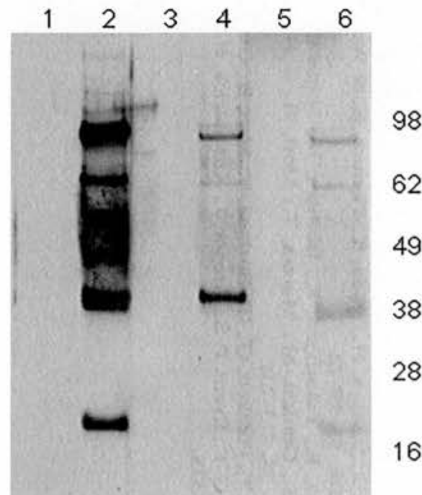
Lanes 1, 3 and 5 loaded with outer membranes and lanes 2, 4 and 6 with cytoplasmatic protein preparations. Both blots were similarly processed and all lanes were loaded with 1 µg of antigen.

Transfers 1 and 2 were incubated with a pool of mucosal supernatant from 3 animals with histopathological lesions, whereas transfers 3 and 4 were incubated with supernatants from animal 1 and lanes 5 and 6 with supernatants from animal 2

Exposure time lane 10 s. Lanes 1 and 2, were exposed for a shorter period due to the relatively high intensity of the reaction

Supernatant proteins from ZAP 198 and a LEE4 deletion mutant (ZAP 984) were prepared as described in section 2.15 of M&M and used to determine if there were detectable IgA responses to type III-secreted proteins. Reactive bands with molecular masses expected of EspA (22/25kD) EspD-B (37/39kD) and Tir (100/110kD) were detected when samples were incubated with pooled rectal tissue extracts. No reactive bands were observed when the same extracts were used to blot supernatant proteins precipitated from the LEE4 mutant (Figure 5-10).

Figure 5-10. Reducing 1-D SDS-PAGE Western blot of *E. coli* ZAP 198 secreted proteins and supernatants of a LEE4 mutant incubated with rectal mucosa extracts obtained at *post-mortem* from group 1 and 2 animals



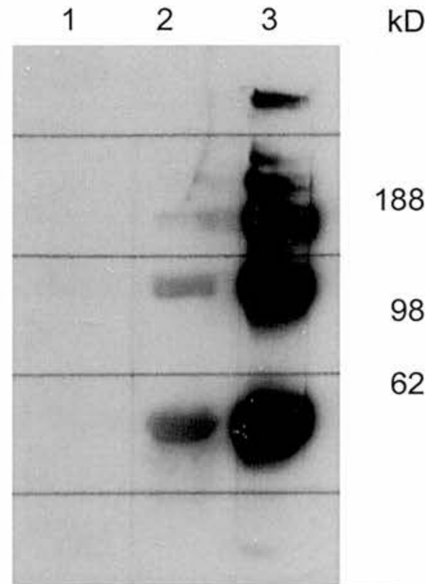
Lanes 1, 3 and 5 loaded with supernatants of a LEE4 mutant and lanes 2, 4 and 6 with secreted proteins.

Transfers 1 and 2 were incubated with a pool of mucosal supernatant from 3 animals with histopathological lesions, , whereas transfers 3 and 4 were incubated with supernatants from animal 1 and lanes 5 and 6 with supernatants from animal 2

Exposure time lane 10 secs. Lanes 1 and 2, were exposed for a shorter period due to the relatively high intensity of the reaction.

The detection of specific *E. coli* O157:H7 antigens was also extended to the flagellum. Flagella are with LPS one of the two antigens (O and H) used for classifying the *E. coli* group. IgA reactivity was also investigated against purified H7 flagellin, the protein that forms the filamentum, and 2-3 bands were visible between 60 and 200 kD on extracts from animal 1. A similar band pattern profile was observed following immunoreaction with a commercial monoclonal antibody against H7 antigen, an example from one animal is shown (Figure 5-11).

Figure 5-11. Reducing 1-D SDS-PAGE Western blot of *E. coli* ZAP 198 H7 flagellin incubated with rectal mucosa extracts obtained at post-mortem from group 2 animals



Lanes 1 to 3 H7 flagellin

Lane 1 no primary, lane 2 case 1-2 and lane 3 rabbit policlonal anti-O157 LPS antibody

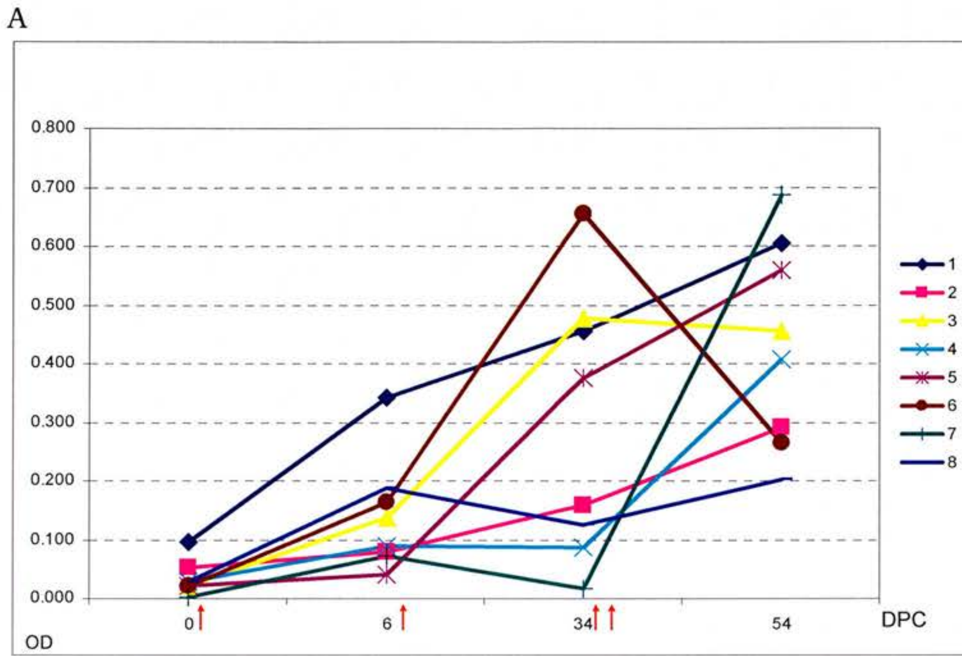
5.3.2 ELISA

To understand better the development of immune responses after infection a qualitative ELISA study was carried out from rectal biopsy samples taken at four different time points during the 50 days of the study, using purified O157 flagellin and LPS as antigens. The assay method is described in section 2.17 of M&M.

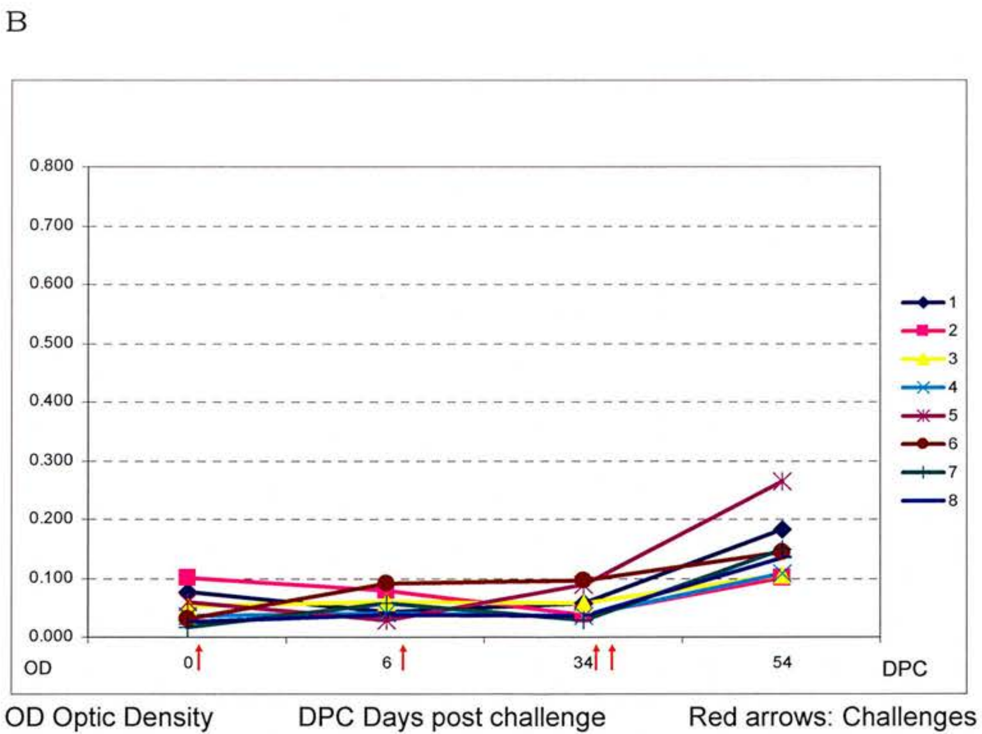
The mucosal antibody responses measured by ELISA showed an elevated mucosal IgA response at day 50 in all animals against both O157 LPS and H7 flagellin. This response was particularly marked to H7 flagellin when compared with LPS (Figure 5-12a and 5-13a).

Figure 5-12. Levels of rectal mucosa H7 specific IgA (A) and IgG1 (B) detected in Group 2 animals after repeated challenges with *E. coli* O157:H7 ZAP 198

A



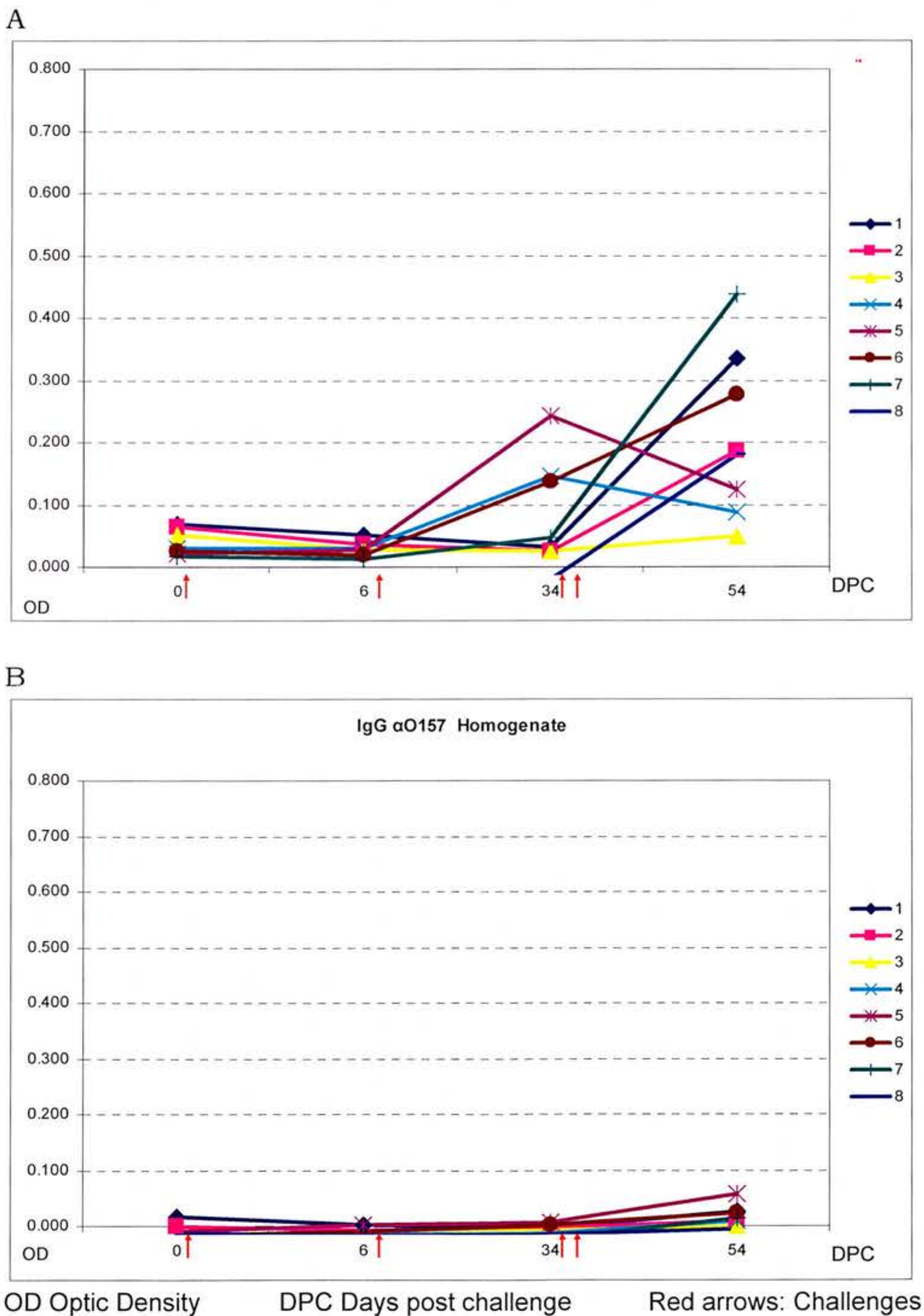
B



OD Optic Density DPC Days post challenge Red arrows: Challenges

Similarly, the readings for IgG1 antibody were generally higher for H7 than for LPS (Figure 5-12b and 5-13b). Overall, the rectal mucosal antibody titres increased after repeated *E. coli* O157:H7 challenge.

Figure 5-13. Levels of rectal mucosa O157 LPS specific IgA (A) and IgG1 (B) detected in Group 2 animals after repeated challenge with *E. coli* O157:H7 ZAP 198.



OD Optic Density DPC Days post challenge Red arrows: Challenges

5.4 Discussion

There have been other investigations of the serological immune responses to *E. coli* O157 (Hoffman et al., 2006; Johnson et al., 1996; Lissner et al., 1996 and other EHEC (Widiasih et al., 2004a) in cattle. However, this study demonstrates for the first time, that *E. coli* O157:H7 infection in cattle induces local IgA immune responses against membrane and secreted proteins antigens at the terminal rectum, the principal site of *E. coli* O157 colonisation in the bovine species.

Immunoblotting showed a number of antigens in whole cell preparations that were recognised by IgA in rectal mucosa extracts collected post-challenge. These bands were more numerous and intensely stained than those detected after reaction with mucosal extracts from pre-challenge tissue, indicating a local IgA antibody response to the repeated *E. coli* O157:H7 challenges. Shared antigens from other *E. coli* present in the normal flora of farm reared calves, or cross reactivity with other antigens (Stenutz et al., 2006; Westerman et al., 1997) are likely to explain the low pre-challenge reactivity observed.

Proteinase K treatment of whole cell preparations removed almost all reactive material, suggesting that a high proportion of the antibody targets were protein rather than carbohydrate epitopes. In agreement with this finding, Western immunoblot analysis of purified O157 LPS showed low reactivity. The lower reactivity of the O157 LPS when compared with protein antigens was also confirmed by ELISA analyses, carried out with equal antigen loads and primary antibody

concentrations. LPS is one of the main pathogen-associated molecular patterns (PAMPs) employed by the mammalian's innate immune system to detect Gram-negative bacteria (Backhed et al., 2003; Trent et al., 2006), although pure LPS is considered a poor immunogen (Poxton, 1995). Thus, in this case LPS does not appear to be a dominant antigen for the development of a mucosal response.

The rectal mucosal extracts labeled three major reactive bands in the outer membrane preparations of *E. coli* O157:H7, while the cytoplasmic preparations reacted weakly, suggesting that the major immunogenic proteins of the bacterium are surface-exposed. It is possible that like most of the PAMPs that interact with the innate immune system (Blasi et al., 2005; Rezaei et al., 2006), the main virulence determinants from non invasive bacterial pathogens may be extracellular virulence factors (Parsot, 2005) or membrane components (Kagnoff, 2006). A recent analysis of an expression library recognised by IgG antibodies from immune calves (Kudva et al., 2006) detected predominantly cytoplasmic antigens. However, the work did not identify type III secreted proteins or Shiga toxin against which specific IgG has been previously reported in cattle (Johnson et al., 1996; Potter et al., 2004; Yokomizo et al., 2002). The fact that membrane proteins were the main antigens detected in this study could be a result of restriction site limitations (Rollins et al., 2005) and/or the different tests employed, as well as the differences between antibody isotype and local vs. systemic responses, could explain the greater relevance given in this work to membrane proteins antigens.

In the present study, culture supernatants containing type III secreted proteins where highly antigenic and reacted strongly with IgA antibodies from infected animals. The proteins recognised were not detected in a LEE4 mutant supernatant and had molecular masses that correlate with: (i) the needle elongation filament protein, EspA; (ii) the pore generating protein complex, EspB/D and (iii) the translocated intimin receptor, Tir. These are all key proteins required for induction of attaching and effacing lesions by *E. coli* O157 and are exposed to the host immune system in the early stages of colonisation (Cornelis, 2006). Serological IgG responses against similar proteins have been reported during bovine vaccine trails following vaccination with type III secretion system proteins (Potter et al., 2004) and in human infection (Li et al., 2000). Recent research has found that these proteins are essential for *E. coli* O157:H7 colonisation of the terminal rectum (Naylor et al., 2005b).

H7 flagellin also elicited strong IgA immune responses, with ELISA values higher than LPS for equal concentrations of antigen and primary antibodies. Unlike LPS, flagellin may activate both innate and acquired immune systems, through the activation of Toll-like receptor 5 and the recruitment of dendritic cells (Honko et al., 2005; Rumbo et al., 2006; Yang et al., 1997). This immundominance of flagellin has also been reported in Crohn's disease patients (Lodes et al., 2004). Moreover, in vitro studies have found that flagellin is the main inducer of the neutrophil chemoattractant interleukin 8, through the activation of intestinal epithelial cell NF-κβ and MAP kinase pathways (Berin et al., 2002), and *E. coli* O157:H7 infection in cattle is characterised by the induction in the rectal mucosa of neutrophil infiltrates. In contrast to *E.*

coli O157:H7 infection in cattle, H7 flagella antibodies have not been detected in the sera of human patients (Chart et al., 1989; Jenkins and Chart, 1999), which may indicate the lack of flagella expression during human infection.

In summary, the present study has shown that *E. coli* O157:H7 colonisation induces a strong local mucosal antibody response in the rectum, which is directed at several membrane-associated and secreted proteins. Since IgA has been selected through evolution as the main protective element of the mucosal humoral immune response (Matsunga and Rahman, 1998), the antigens detected may represent important vaccine candidates. However, further work is required to determine their relevance in protection.

Chapter 6

Mucosal antibody responses to *Escherichia coli* O157:H7 outer membrane porin C

6.1 Introduction

The current study has indicated that infection of cattle with *E. coli* O157:H7 induces strong mucosal immune responses in the rectum to different protein fractions of the bacteria. One-dimensional polyacrylamide gel electrophoresis (1D-PAGE) offers very low resolution of fractionated whole *E. coli*, a complex sample that can contain thousands of putative antigens (Tonella et al., 1998; Tonella et al., 2001). The strength of 2-D SDS/PAGE is its ability to separate the proteins present in a sample in two dimensions by both charge and molecular weight. This can increase the resolution of single proteins in complex samples. Further immunoblotting and analysis of the immunogenic spots by peptide mass spectrometry methods allows the identification of individual antigens (Lilley et al., 2002). Despite well known limitations, such as spot overlapping (Campostrini et al., 2005), 2-D SDS/PAGE is still the method of choice for the analysis of complex protein samples (Gorg et al., 2004).

Membrane preparations contained some of the most immunogenic *E. coli* O157 proteins detected in the earlier work. Gram negative bacterial membranes are composed of two membranes, a cytoplasmic and an outer membrane, with the peptidoglycan-containing periplasm situated between these (Duong et al., 1997). The distinct Gram negative outer membrane is composed of a phospholipid bilayer formed by an inner leaflet of phospholipids and an outer leaflet of LPS (Raetz and Whitfield, 2002). This lipid portion of the outer membrane is largely impermeable to all charged molecules (Nikaido and Vaara, 1985). Unique to Gram negative bacteria, channels called porins are present in the outer

membrane to facilitate nutrient transport and these constitute the most abundant proteins in the cell membrane (Doerrler, 2006). These porins account for nearly half of the total mass of the outer membrane (Osborn et al., 1972). Other proteins found in cell membranes are hydrolytic enzymes and binding proteins in the periplasm (Ruiz et al., 2006) and lipoproteins anchored predominantly to the outer membrane but facing into the periplasm (Tokuda and Matsuyama, 2004).

Of the outer membrane proteins the most abundant when bacteria are grown in conventional media are OmpA, OmpF and OmpC (Nikaido, 1996). These porins are composed of eight (OmpA) or sixteen (OmpC and OmpF) anti parallel β-strands that transverse the outer membrane folded in a characteristic β-barrel structure surrounding a channel (Basle et al., 2006; Sansom, 1999). The loops connecting membrane-traversing β-strands are between one to four residues long on the periplasmic side but often longer on the transmembrane external side. The third external loop folds inward narrowing the lumen of the β-barrel in OmpC and OmpF porins (Nikaido, 2003).

The external loops show much variability among enterobacteria (Nikaido 1996). Importantly they could be specific epitopes recognised by the adaptive immune response. Specificity has been confirmed by abrogation of the ability of the porins to serve as phage receptors by introducing mutations into the external loops of OmpA (Morona et al., 1984) and OmpF (Traurig and Misra, 1999), *E. coli* O157:H7 OmpC-specific phages have been reported on several occasions (Morita et al., 2002; Yu et al.,

1998), although no studies have been carried out to define the site of phage attachment.

6.2 Aims and objectives

1- To identify within the whole *E. coli* O157:H7 proteome the most antigenic proteins.

2- Following identification of OmpC as a putative immunodominant protein, to construct an *ompC* mutant and confirm loss of antigen recognition.

3- To define the transmembrane external epitopes of OmpC that are recognised by the bovine host.

To achieve these objectives the following approaches were taken:

Optimisation of a 2-D SDS-PAGE gel electrophoresis and immunoblotting system for whole *E. coli* O157 preparations.

Identification of immune-reactive proteins by MALDI-TOF/TOF mass spectometry analysis

Construction of an *ompC* mutant by allelic exchange followed by Western blotting of the mutant to confirm loss of antigen recognition.

Peptide synthesis of the most relevant external structures of OmpC identified from a published structure.

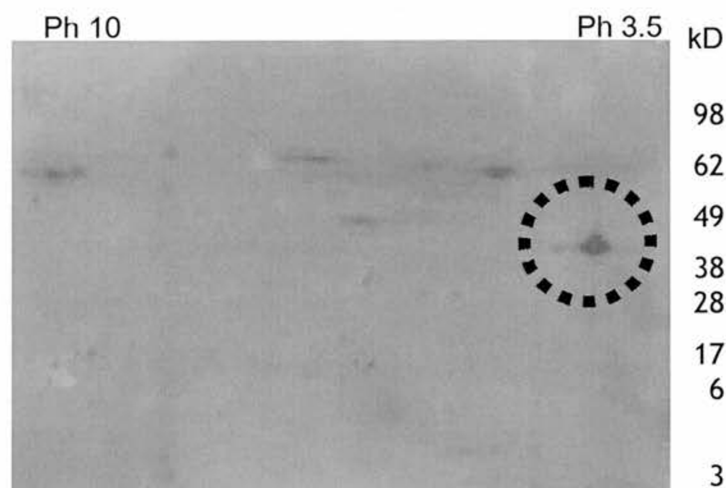
Western blotting and ELISAs to probe for antibody recognition of the OmpC peptides.

6.3 Results

6.3.1 Antigenicity analysis of the *E. coli* O157:H7 ZAP 198 proteome by 2-D gel electrophoresis and Western blotting

Western blot analysis of transfers of 2-D SDS-PAGE (Section 2.21 M&M) of antigen preparations of whole *E. coli* O157:H7 strain ZAP 198, allowed the detection of IgA antibodies against one spot identified by mass spectrometry analysis (score 131, significance level 60) as outer membrane porin C (OmpC). IgA antibody from mucosal tissue homogenates collected from ten animals tested (animals 204, 722, 868 from group 1 and 7calves from group 2), all detected the OmpC protein albeit with varying degrees of reactivity. A representative example is shown below (Figure 6-1).

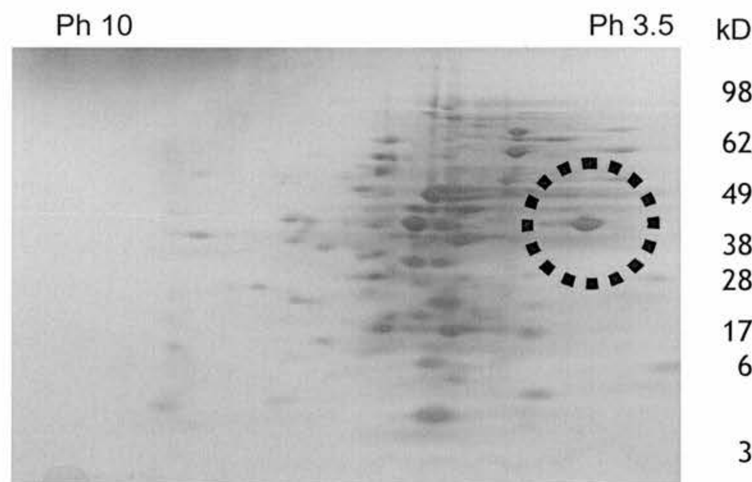
Figure 6-1. Reducing 2-D SDS/PAGE Western blot analysis of E. coli ZAP 198 incubated with rectal mucosal extracts (case 8, group 2) showing a reactive spot later identify by mass spectrometry analysis as OmpC (dotted circle). Exposure 15s biotin conjugated tertiary antibody.



Although OmpC reacted strongly and was readily detected by MALDI-TOF/TOF, several other weaker reactive spots were observed. However, despite several attempts at identifying these through MALDI-TOF/TOF, no significant identities were established.

Four 2-D gels were employed to separate the proteins and the gels were stained with Simply Blue and matched with the immuno reactive spot observed on the counterpart Western blots. The gel spots were excised and identified by mass spectrometry analysis (score 131 ±3, significance level 60) as outer membrane porin C (OmpC) (Figure 6-2).

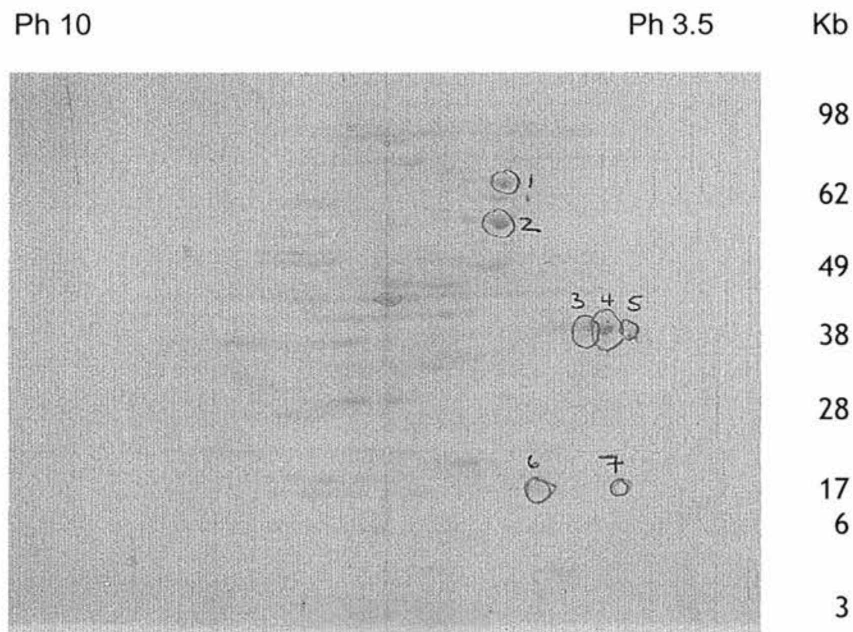
Figure 6-2. Reducing 2-D SDS/PAGE analysis of *E. coli* ZAP 198 proteonome, showing several spots of Simply Blue positive stained material. OmpC marked with a dotted circle



Confirmation that OmpC had focused on a single spot in the gel was sought by sampling collateral areas and no match was found with any peptide sequence. Sampling of another area where immunoblots from three cases had presented an immunoreactive spot of about 17 kD did

not match any protein spot on the 2-D SDS/PAGE gels. Another three additional gel spots matching proteins DNAK, Cpn 60 and Ferritin were sampled to verify the correct distribution of *E. coli* proteins in the 2-D SDS/PAGE gel (Figure 6-3).

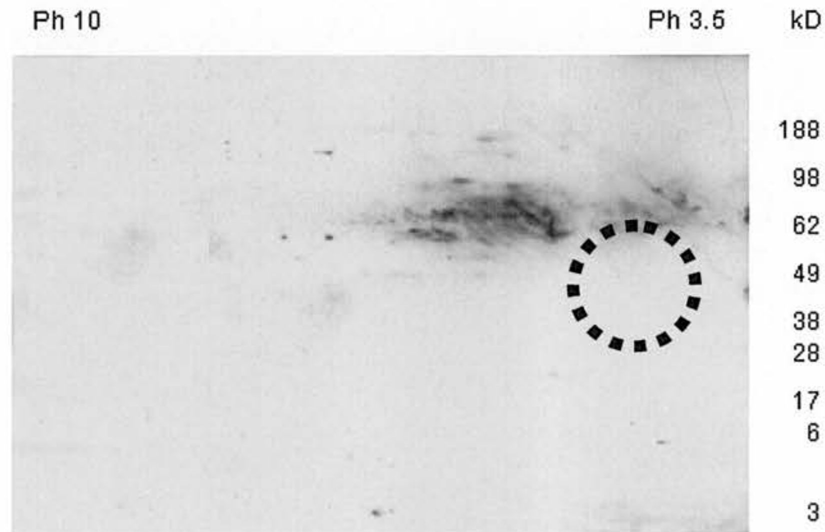
Figure 6-3. Reducing 2-D SDS/PAGE analysis of *E. coli* ZAP 198. The protein spots marked with a circle were sampled for Maldi-TOF/TOF mass spectrometry analysis



Spot 3: no match. Spot 4: Outer membrane protein C; Spot 5: no match; Spot 7: no match; Spots 1, 2 and 6 DNAK, Cpn 60 and Ferritin respectively.

To evaluate the specificity of the immune response to *E. coli* O157 OmpC, K-12 was analysed by 2-D Western blot. Immunobloting did not detect any IgA reactive peptide spots on the OmpC area when the K-12 strain was incubated with a pool of group 1 mucosal extracts (Figure 6-4).

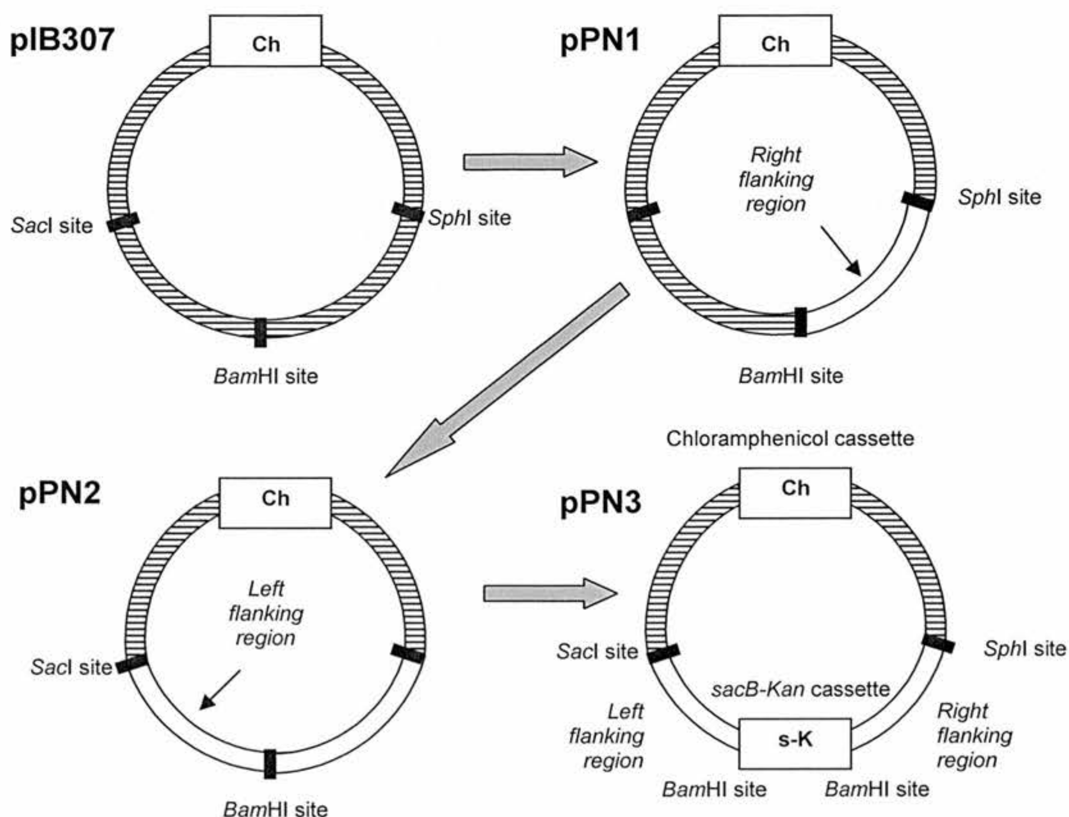
Figure 6-4. Reducing 2-D SDS/PAGE Western blot analysis of *E. coli* ZAP 185 incubated with rectal mucosal extracts from a pool of three animals from group 1 (dotted circle, expected area of OmpC spot identification). Exposure 15 s biotin conjugated tertiary antibody.



6.3.2 Targeted mutagenesis of *ompC*

To eliminate the possibility of insufficient separation and interference from another protein present at levels below mass spectrometry detection threshold, it was decided to construct an *ompC* mutant for further immunoblot testing. The main steps followed for this process were outlined in the section 2.42 of the M&M and are also represented in Figure 6-5.

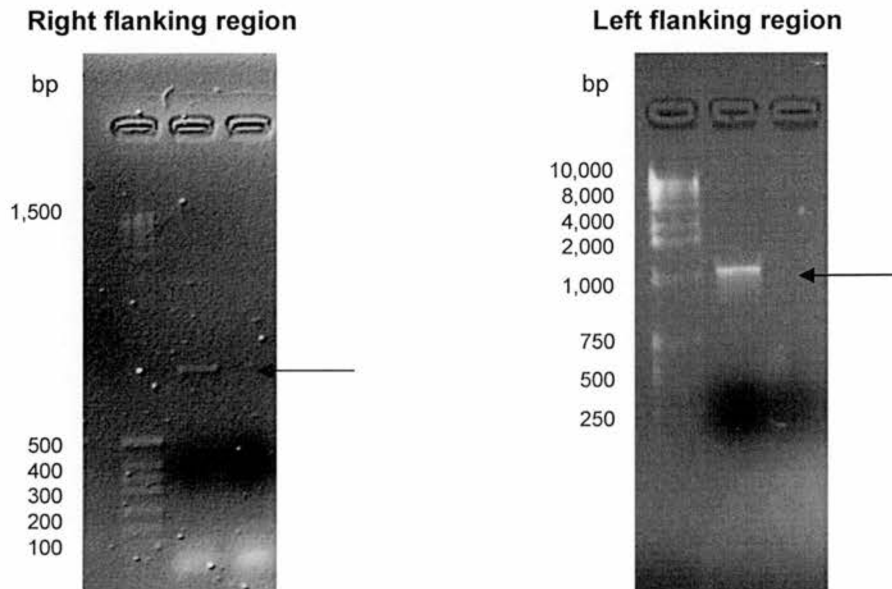
Figure 6-5. Schematic diagram of the sequential steps of plasmid construction



Plasmid construct

Amplification of homologous regions. Using the primers described in section 2.27 of M&M both *ompC* flanking regions of 1,000 and 1,113 bp were amplified from an *E. coli* O157:H7 ZAP 198 DNA template, and the correct final PCR products identified by their size by agarose electrophoresis. The primers incorporated additional *BamH*I, *Sph*I and *Sac*I restriction sites to facilitate sub-cloning into the corresponding restriction sites in pIB307 (Figure 6-6).

Figure 6-6. PCR amplification of right (1,000 bp) and left (1,113 bp) flanking regions using *E. coli* ZAP 198 DNA as template. First lane marker, second amplified product (arrow), third no template.

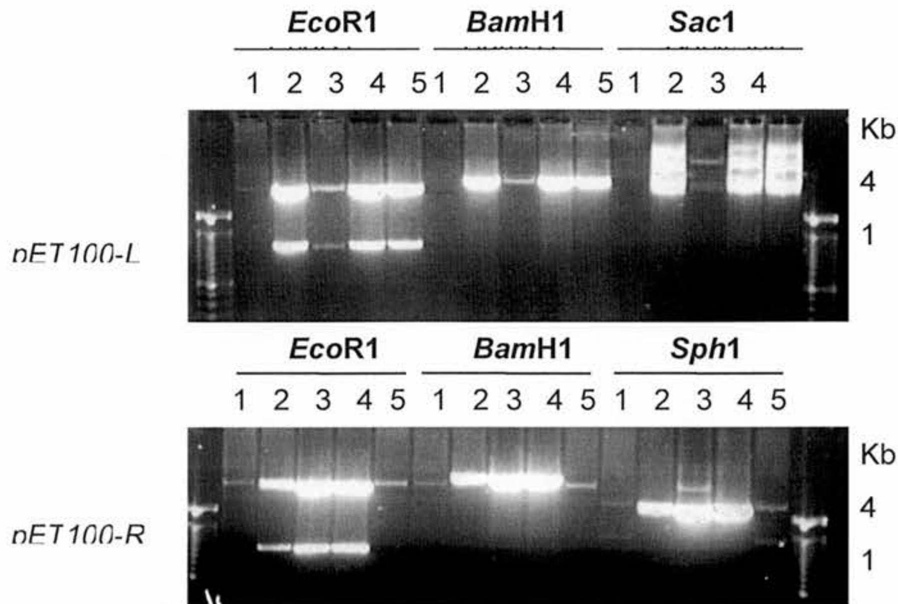


Cloning of the left and right inserts into pET100 and transformation of the new plasmids pET100–R and pET100–L into host bacterial cells

After several failed attempts to introduce directly the right flanking region into pIB307, it was decided to clone each fragment first into a TOPO pET100 plasmid for further excision and insertion into pIB370. This strategy facilitates cloning for two reasons. First it takes advantage of the higher copy number of this replicon. Secondly the addition during the PCR of a single 3'-A overhang to each end of the products enables more efficient cloning into the TOPO vector, that is prepared with 3'-T overhangs. After 3'-A overhang amplification, both homologous regions were cloned into two different sets of TOPO pET100 plasmid (sections 2.24 and 2.36 M&M). The new plasmid constructs were introduced into two different preparations of K-12 S185 host cells by heat shock transformation (section 2.38 M&M) and plated onto LBA plates. For each

set of transformants five colonies were selected and cultured overnight in LB broth. Plasmids were purified (section 2.25 of M&M) and submitted to diagnostic digests (one hour at 37°C) with the following sets of enzymes: *EcoR1* a restriction site situated at both ends of the cloning site, *Bam*H1/*SpH*1 for the right flanking region and *Bam*H1/*Hind*III for the left region. The size of the products expected was 3.9 Kb for the *EcoR*1 digestion with a second band containing the insert, and 1.0 and 1.1 for right and left hand inserts respectively. 2 μl of the samples were run on a 1% agarose gel for one hour at 100 v. Several clones showed DNA products of the size of the flanking regions, and clone 2 for the left hand region and clone 3 (lanes 2 and 3, Figure 53) for the right hand region were selected for subsequent sub-cloning into pIB307. The new plasmids containing the right and left flanking regions were designated pET100–R and pET100–L respectively (Figure 6-7).

Figure 6-7. Diagnostic digestions of left (pET100-L) and right (pET100-R) TOPO pET100 plasmid construct purified from five different clones

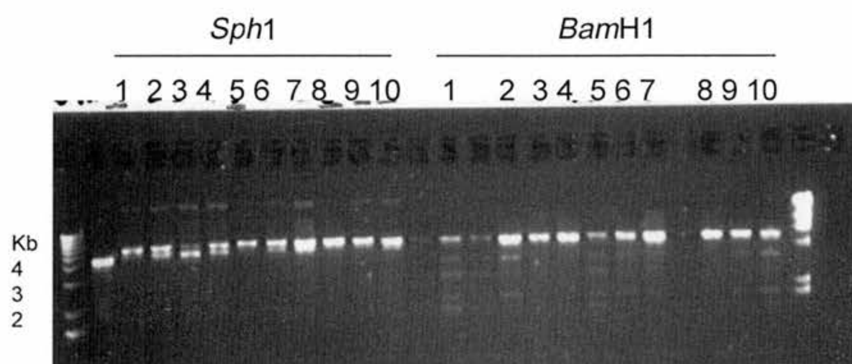


Subcloning of the left and right inserts into pIB307.

To subclone the right flanking region from pET100–R into pIB307, plasmid purified preparations were submitted separately to consecutive digests with *SphI* and *BamHI*. Insert DNA was identified by its molecular size using agarose gel electrophoresis, excised and the DNA extracted as previously described (section 2.34 M&M). The vector was then ligated with digested pIB307. The proportion of DNA of vector to insert present on the gel was estimated at a ratio of 1:3 and a similar proportion was used for the ligation, which was performed as described in section 2.35 of the M&M. Finally putative plasmids containing the right flanking region were introduced into *E. coli* competent cells by heat shock transformation (section 2.38 M&M) and plated onto LBC plates. Ten colonies were then cultured overnight and plasmids extracted and digested. The expected DNA size for the new plasmid construct was 5.2 Kb (pIB307 and right

flanking region DNA sizes 4.2 Kb and 1 Kb respectively) (section 2.24, table 2-7 M&M). Confirmation of successful inserts was obtained after digestion of the preparations with either *Bam*HI or *Sph*I. The new plasmid containing the right flanking region was designated pPN1 (Figure 6-8).

Figure 6-8. Diagnostic digestion with *Sph*1 and *Bam*HI of plasmid construct pPN1 and right flanking region purified from ten different clones

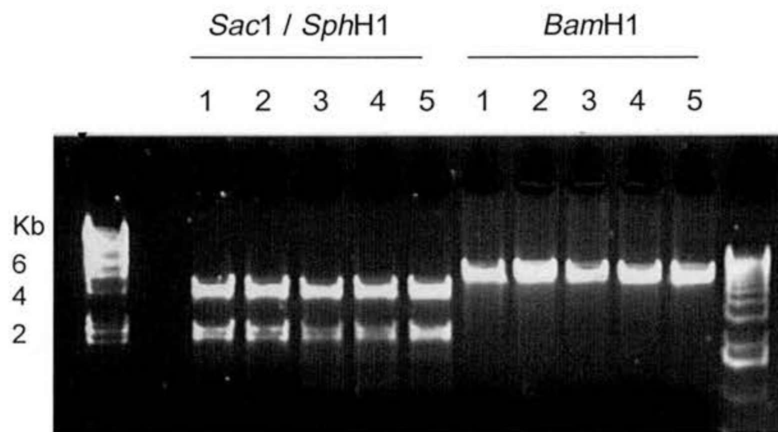


Estimated product size 5.2 Kb

To insert the left flanking region into pPN1, plasmid purified preparations of pET100–L and pPN1 clone 8 (Figure 6-8, lane 8), were submitted separately to consecutive digestions with *Sac*I and *BamH*I. The DNA of the vector and insert were identified by molecular size using agarose gel electrophoresis, the bands excised, and the DNA extracted as described in section 2.34 of the M&M. The proportion of insert DNA to vector was estimated at 1:3 and a similar proportion was used for the ligation, which was performed as described in section 2.35 of the M&M. The ligation was then introduced into *E. coli* competent host cells by heat shock transformation (section 2.38 M&M). Five transformants were selected and cultured overnight in LB broth, the plasmids were purified and two aliquots of 2 µl submitted to overnight digestion with *Bam*HI alone and

with a combination of *SacI* and *SphI* enzymes (Figure 6-9). The products obtained were of the expected size (pPN1 and left flanking region DNA sizes 5.2 Kb and 2.1 Kb respectively) (section 2.24, table 2-7 M&M), and the new plasmid containing the right flanking region was termed pPN2 (Lane 5, Figure 6-9).

Figure 6-9. Diagnostic digestion with *Bam*H1 alone and a combination of *SacI* and *SphI* of plasmid construct pPN2 with right and left flanking regions purified from five different clones

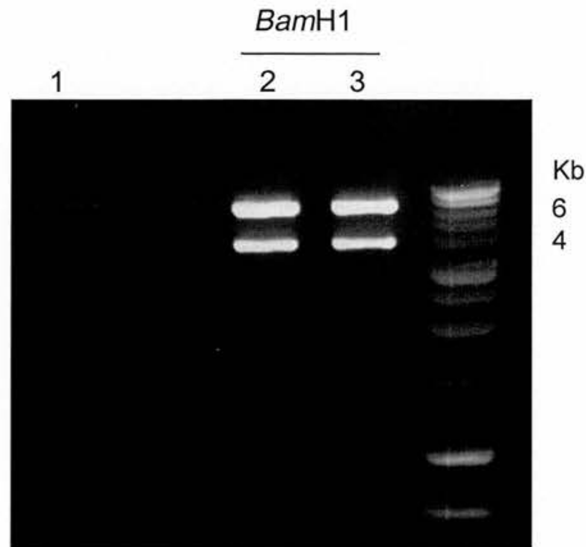


Estimated total plasmid size 6.4 Kb, backbone 4.2 Kb, insert 2.1 kb

Purification and cloning of the *sacB-Kan* cassette to form pPN3

The construct pPN2 from preparation 5 was digested with *Bam*HI and the resultant linear DNA de-phosphorylated with SAP (1.1.1.13). The *sacB-kan* cassette was extracted from vector pKC11.04 by restriction digestion with *Bam*HI and gel purified from two different clones. Insert and vector were ligated, transformed into calcium competent cells and the new construct termed pPN3 confirmed by diagnostic digestion with *Bam*HI (Figure 6-10).

Figure 6-10. Diagnositc digestion with *Bam*HI of plasmid construct pPN3 from two different clones containing the *sacB-Kan* cassette

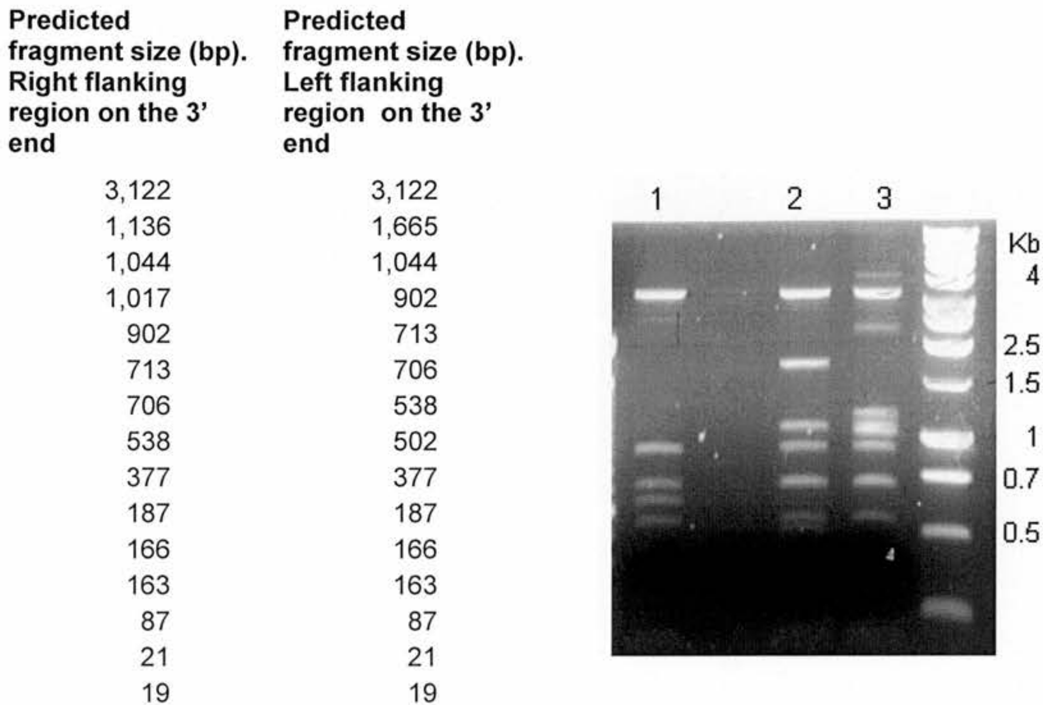


Estimated total plasmid size 10.2kb, backbone 6.4 Kb and *sacB-Kan* 4 kb, lane 1 parent plasmid, lanes 2 and 3 pPN3 digestions.

Elucidation of *sacB-Kan* position

The vector pPN3 obtained from the two clones was cleaved with the enzyme *RsaI* for which there should be 15 sites in the vector and insert. The insert could have been spliced into the plasmid in one of two orientations. The expected size of the fragments for each configuration is listed in Figure 57. Both orientations were obtained, the clone in lane 3 had the insert situated with the left flanking region on the 3' end and clone number 1 on the 5' end (Figure 6-11). The clone analysed in lane 3 was used in the ensuing allelic exchange.

Figure 6-11. Predicted fragement sizes and resulting diagnostic digestion of*sacB-Kan* position following cleaveage of the vector pPN3 with the enzyme *Rsa*I.



Predicted fragment size (bp). Right flanking region on the 3' end	Predicted fragment size (bp). Left flanking region on the 3' end
3,122	3,122
1,136	1,665
1,044	1,044
1,017	902
902	713
713	706
706	538
538	502
377	377
187	187
166	166
163	163
87	87
21	21
19	19

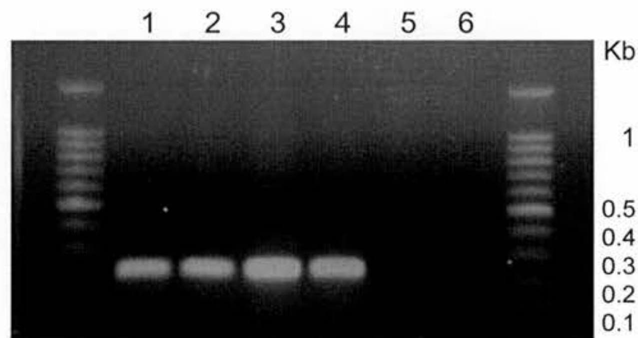
Lane 1 parent plasmid, lanes 2 and 3 pPN3 digestions.

Transformation into *E. coli* O157:H7 electro-competent cells and sub-culturing

To replace in the host chromosome *ompC* with the *sacB-Kan* cassette via homologous recombination, clone 3 was transformed into *E. coli* O157:H7 ZAP 198 electro-competent cells following the protocol described in section 2.39 of M&M. After selection for plasmid integration by sub-culturing repeatedly in LBC broth, followed by similar sub-culturing in LBK broth to select for plasmid excision (section 2.42 M&M, Figure 2-7), bacterial colonies were screened for transformants in which a secondary recombination had induced the replacement of the *ompC* gene for the *sacB-Kan* cassette. Serial dilutions (10^{-3} to 10^{-6}) were plated and 300 colonies patched on both LBC and LBK plates. Ten clones that grew on

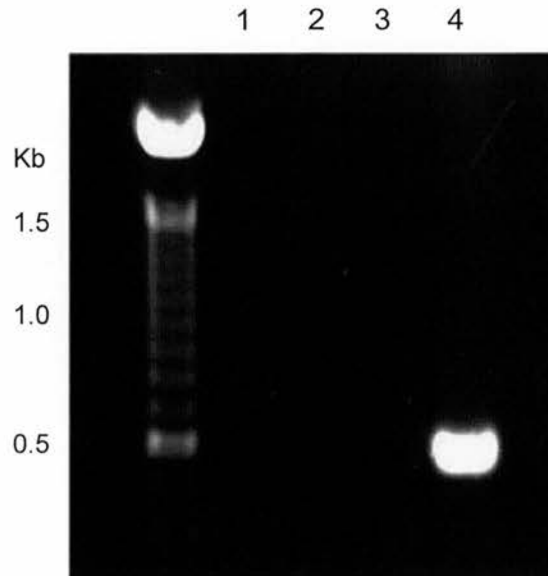
LBK but not on LBC were tested by PCR for the absence of the vector pIB307 and *ompC*, and presence of an area comprising the right flanking region and a nearby downstream area. PCR amplification confirmed that *ompC* had been successfully replaced with the *sacB-Kan* cassette in a number of colonies tested (Figure 6-12). The clone analysed in lane number 1 (Figure 6-12) was selected and further PCR amplifications were carried out to confirm the absence of the parent plasmid pIB307 (Figure 6-13) and the replaced gene *ompC* (Figure 6-14). This clone was used thereafter for proteomic analysis and Western blotting.

Figure 6-12. Results of the PCR amplification of the *sacB-Kan* cassette from three *E. coli* ZAP 198 transformant clones



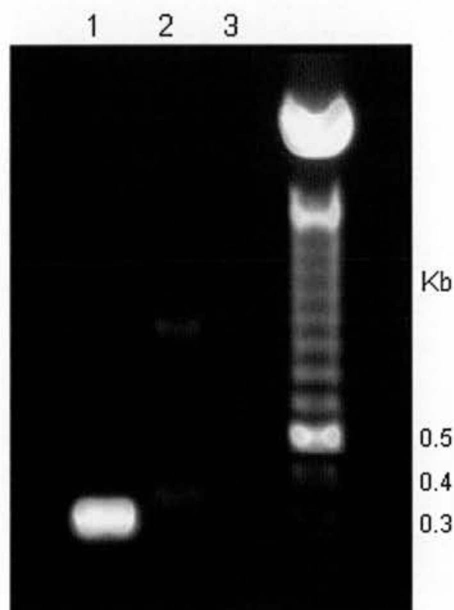
Lanes 1, 2, 3 transformant clones; lane 4 pPN3, lane 5 *E. coli* ZAP 198; lane 6 no-template.

Figure 6-13. Results of the PCR amplification of the pIB307 backbone from *E. coli* ZAP 198 transformant clone 1



Lanes 1 clone 1; lane 2 *E. coli* O157:H7 ZAP 198; lane 3 no-template; lane 4 pIB307 preparation.

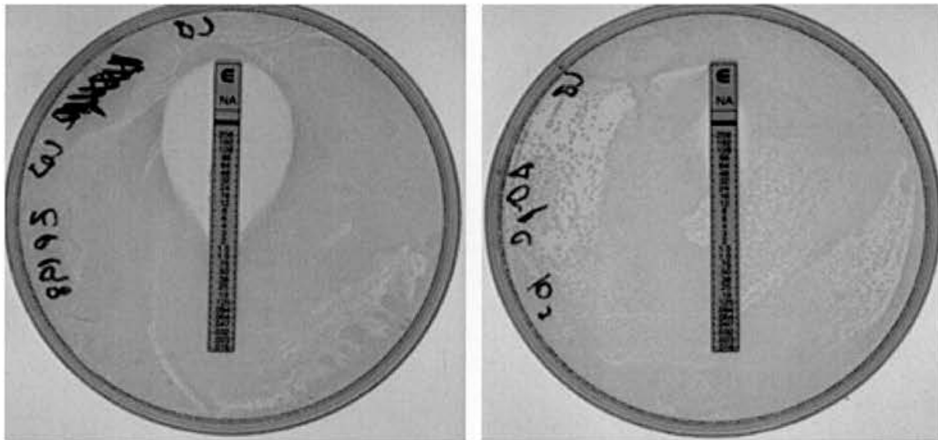
Figure 6-14. Results of the PCR amplification of the *ompC* gene from *E. coli* ZAP 198 transformant clone 1



Lanes 1 clone 1; lane 2 *E. coli* O157:H7 ZAP 198; lane 3 no-template

Finally, an agglutination test (section 2.3 M&M) was carried out on the mutant strain and *E. coli* ZAP 198 and both were confirmed as *E. coli* O157:H7. A similar phenotype for both strains was observed in preparations examined by light microscopy, although a marked resistance to the quinolone antibiotic nalidixic acid was observed in the *ompC* mutant strain when using E-test strips (Figure 6-15). The strain was termed *E. coli* ZAP 1187.

Figure 6-15. Minimum inhibitory concentrations of nalidixic acid observed on strains *E. coli* ZAP 198 (left) and *E. coli* ZAP 1187 (right).



6.3.3 2-D SDS/PAGE analysis of *E. coli* ZAP 1187 and *E. coli* ZAP 198 proteome

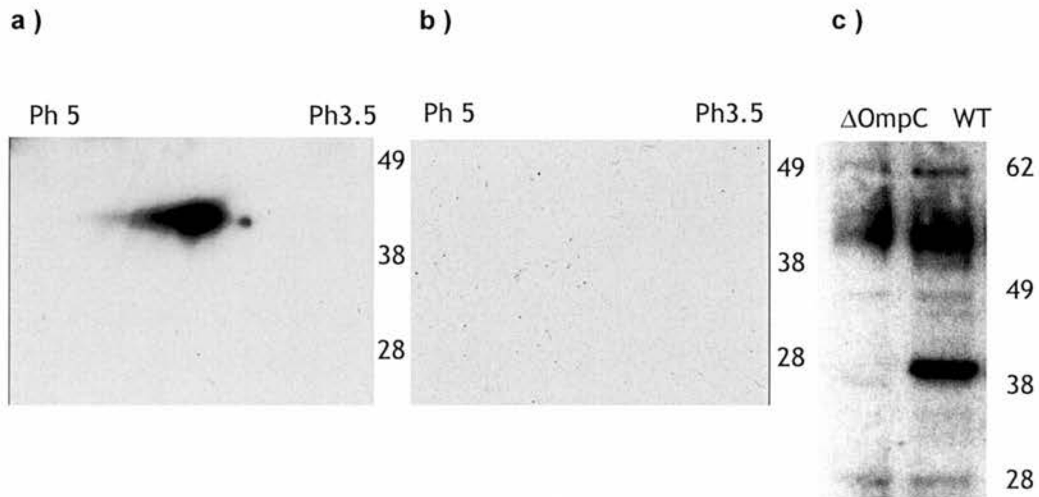
Two-dimensional gel electrophoresis and Maldi ToF-ToF mass spectrometry analysis of *E. coli* ZAP 198 identify OmpC on the wild type strain, while *E. coli* O157:H7 ΔOmpC *E. coli* ZAP 1187 proteome revealed the absence of the OmpC spot in the same are of the gel (Figure 6-16).

Figure 6-16. 2-D SDS/PAGE Simply Blue stained proteome profile of *E. coli* ZAP 198 and *E. coli* ZAP 1187. OmpC is marked with a dotted circle on the parent strain *E. coli* ZAP 198, while is absent in the mutant *E. coli* ZAP 1187 strain.

6.3.4 Western blot detection of mucosal IgA antibodies against *E. coli* ZAP 198 and 1187

An immunoblot of the OmpC deletion mutant strain *E. coli* ZAP 1187 did not detect any IgA reactive peptide in the OmpC region, while the wild type (WT) *E. coli* ZAP 198 did react. Incubation was carried out with homogenates with the same pool of mucosal extracts from group 1. 2-D Western blotting analysis showed the IgA reactive protein OmpC (Figure 6-17a). Immunoblot analysis of the mutant strain (ΔOmpC) indicated the absence of OmpC (Figure 6-17b). Similarly, 1-D SDS immunoblot analysis of outer membrane preparations of the parent WT and ΔOmpC strain showed a reactive band in the WT preparation of 42 kD region which was absent in the ΔOmpC preparation (Figure 6-17c). Thus, confirming that the originally identified immunogenic protein was no longer detectable in the ompC mutant.

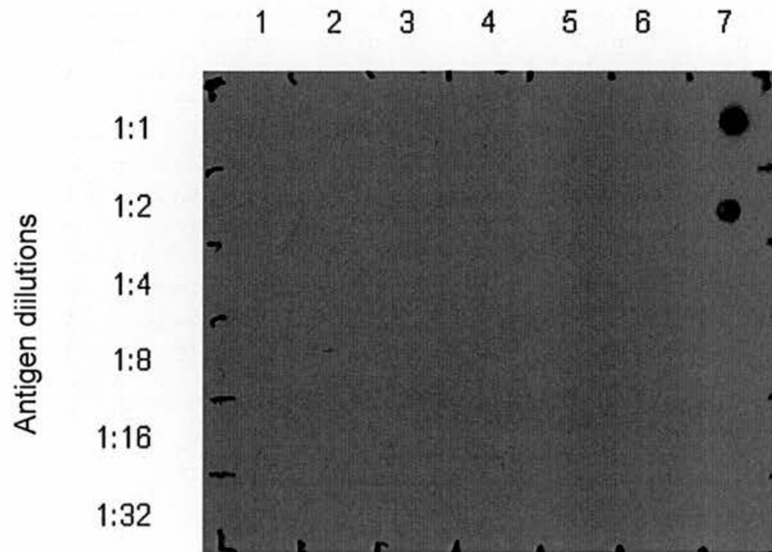
Figure 6-17. Reducing SDS-PAGE/Western blot analysis of *E. coli* ZAP 198 and mutant strain ZAP 1187. (a) 2-D immunoblot of *E. coli* ZAP 198 showing the IgA reactive protein OmpC; (b) 2-D immunoblot analysis of *E. coli* ZAP 1187 indicating the absence of OmpC reactivity; (c) 1-D SDS immunoblot analysis of outer membrane preparations of E. coli ZAP 198 and ZAP 1187. Exposure 15s biotin conjugated tertiary antibody.



6.3.5 Immunoblotting and ELISA testing of putative OmpC immunogenic peptides

The peptides of the external hairpin loops of OmpC failed to elicit any immunoreactivity by dot blotting (Section 2.18 of M&M) or ELISA (Section 2.17 of M&M) on the external loops 1, 2, 4, 5 and 6 of OmpC (Section 2.40 of M&M) incubated with nasal secretions, mucosal extracts and sera from five animals of Group 4. These animals were selected for their high ELISA titres to *E.coli* O157:H7 flagellin antigen. A representative sample of one of the blots is presented in Figure 6-18.

Figure 6-18. Dot blot analysis of the immunoreactivity of OmpC external loops



Antigen blotted by columns in serial dilutions, as follows: column 1 loop 1, column 2 loop 2, column 3 loop 4, column 4 loop 5, column 5 loop, column 6 negative control, column 7 *E. coli* ZAP 198. Dotted proteins incubated with the mucosal extracts (0.3 mg/ml of protein)

6.4 Discussion

Following 1-D SDS-PAGE/Western blot analysis, further attempts to define the reactive antigens was by 2-D SDS-PAGE/Western blotting. Mass spectrometry analysis identified OmpC as a bacterial membrane antigen detected by the IgA mucosal response. The immunodominance of this protein has been demonstrated for patients with Crohn's Disease where *E. coli* OmpC has been proposed as a reliable marker to characterise infection (Spivak et al., 2006) and in *Salmonella enterica,* for which there is a stronger response to OmpC compared with the other major outer membrane porin, OmpF (Secundino et al., 2006).

The marked difference observed in the number of antigens detected by 1-D and 2-D Western blotting may be due to differences in sensitivity, denaturation (Harper et al., 1990) or to the reported absence in the 2-D gels of some antigens (Madden et al., 2002). Membrane proteins and in particular integral proteins that span the lipid bilayer such as porins, are difficult to solubilise in the media used for isoelectric focusing and are reportedly underrepresented on 2-D gels (Santoni et al., 2000). Membrane proteins are soluble in lipid bilayers and not in water and are very sensitive to different processing methods (Bunai and Yamane, 2005). Changes in solubility or conformation, could explain the detection of Transaldolase B instead of OmpC, when sample cleaning products were intentionally avoided. This enzyme has also been identified in the serological responses of immune calves (Kudva et al., 2006).

Complete separation of proteins is still far from being achieved in 2-D polyacrylamide gel electrophoresis and more than one protein can be present at the same gel spot (Campostrini et al., 2005). Advanced separation methodologies such as sample pre-fractionation (Gorg et al., 2004) or subcellular fractionation (Stasyk and Huber, 2004) may provided a more sensitive means of protein separation of complex mixtures and facilitate immune detection. In this study, to avoid the possibility of the identification method (mass spectrometry) being less sensitive than the detection method (Western blot) and to confirm the immunogenicity of OmpC, it was decided to generate an *ompC* knock out strain Δ*ompC*. The ensuing 2-D analysis showed that the Δ*ompC* strain, while having a similar proteome to the parent strain, was not expressing the protein nor eliciting any reactive spot around the OmpC isoelectric and mass area, thus confirming its specific antigenicity.

The finding that the ΔOmpC strain had increased resistance to nalidixic acid is not in agreement with the few published works on this area. In one study, resistance to nalidixic acid was linked exclusively to OmpF mutants (Hirai et al., 1986), these authors also noted increased resistance to the nalidixic acid in lipopolysaccharide deficient mutants and suggested the passage of this highly hydrophobic quinolone through OmpF and the lipid bilayer. Their hypothesis was that quinolones act as membrane chelating agents (Chapman and Georgopapadakou, 1988). However, a more recent study did not find any changes in MIC levels in OmpF and OmpC single and double mutant strains (Mitsuyama et al., 1992). This reasearch was carried out with the laboratory strain *E. coli* K:12 that may have differences in antibiotic permeability compared to the

E. coli O157:H7 strain, used in the present study and which originated from a human clinical isolate. Further antibiotic resistance testing with the OmpC deficient mutant and an OmpC complemented strain could provide further evidence in support of this preliminary finding.

Unfortunately, there was no evidence of antigenicity to the external loops as determined by dot blot and ELISA. It is generally accepted that these structures are constantly changing due to the selective pressure of the host immune system (Low et al., 2001; Nikaido, 2003) and OmpC external loops are recognised epitopes in *Salmonella typhimurium* infection of mice (Singh et al., 1996). Nevertheless, other studies suggest that in *E. coli* these external loops are covered by the surrounding lipopolysaccharide (Bentley and Klebba, 1988) and that most of the porin antigens are buried (Singh et al., 1996). Other reasons that could explain the lack of immunoreactivity to the peptides are conformational differences between the synthesised products and their real presentation shape as hairpins between beta sheets (Klebba et al., 1990), or divergences between the sequences obtained from the public databases for *E. coli* O157:H7 used for peptide synthesis and the experimental strain. The detection methods used in this work could have sensitivity limitations that may preclude the detection of immune responses. Also, due to the absence of tested candidates, it was not possible to use a synthetic peptide as positive control. Nevertheless the amount of purified protein used to coat or impregnate the matrix was considerable in relation with known amounts or protein commonly used for these tests, and similar assays with synthetic peptides and loads have been carried out by a wide range of researchers (Ankelo et al., 2007; Tantrawatpan et

al., 2007; Sun et al., 2007). Specific antibodies could have been isolated by affinity chromatography,

Chapter 7

General Discussion

It is important to fully understand bacterial colonisation mechanisms and host-parasite interactions to be able to develop methods of preventing bacteria from initiating an infectious process. It was not clear at the initiation of this work whether the colonization ability of *E. coli* O157:H7 depended on underlying mechanisms of bacterial adhesion and whether host immune responses developed following colonization. Evidence produced during the investigation presented in this thesis demonstrates for the first time the following four findings: (i) the rectal challenge route induced similar qualitative and quantitative patterns of colonization at the terminal rectum to the oral route; (ii) bacterial intimate adhesion to bovine rectal epithelium induces histopathological changes, similar to those described elsewhere in the intestinal tract, characterised mainly by severe alteration of the enterocyte apical surface; (iii) rectal colonisation generates a quantifiable granulocytic response, and finally (iv) colonisation leads to the production of a local immune response in the terminal rectum predominantly against protein antigens. The present study also confirms in a higher number of animals previous seminal work carried out by our group demonstrating that *E. coli* O157:H7 has a tropism for the terminal rectum.

The present study has two limitations: first the bolus administration of *E. coli* O157:H7 and second the use of an Stx-negative strain. Therefore, the evidence provided in this study needs to be weighed considering the validity of results obtained from our experimental model.

In this work the infection model used high single doses and repeated doses to ensure maximum colonization rates, although during natural

infection animals are presumably exposed to repeated lower doses of bacteria from the environment, at different ages and in some cases following previous episodes of colonization (Murphy et al., 2008; Shere et al., 1998). Trickle dose (multiple smaller doses over time) would probably have mimicked natural exposure conditions better, but it has not been used before in experimental infections and its efficacy is still uncertain. Indeed, even though high-bolus doses may favour higher colonization rates, it did not induce higher shedding levels than those recorded for naturally infected animals (Chase-Topping et al., 2007; Cobbold et al., 2007), suggesting that, once the colonization is established, infection follows a very similar course in terms of intensity and duration in both scenarios. Thus, the shedding patterns and pathological changes described in chapters three and four for experimentally challenged animals may not be substantially different from the course of the disease in naturally infected animals. Furthermore, histopathological examination of two naturally-infected animals, one of them originally purchased for this study and a steer described previously (Naylor et al., 2003a; Naylor et al., 2005b), detected similar epithelial lesions and granulocytic influx to those observed in the experimental group.

The other methodological characteristic of this work was that the challenge strain ZAP 198 had been naturally cured of the shigatoxin gene. Such a distinctive genotypic alteration could have influenced the colonisation fitness of the bacteria by altering its ability to evade host immune responses, thus affecting colonisation ability or persistence. Previous work by our group had established that ZAP 198 was as effective in colonising calves as its shigatoxin positive parent (Naylor et

al., 2003a), and recent work by other groups with Stx isogenic deletion mutants has corroborated that these strains colonised like the wild type confirming the lack of requirement for Stx in the colonisation of cattle by *E. coli* O157:H7 (Sheng et al., 2006a) and sheep (Woodward et al., 2003; Cornick et al., 2007). Although in human infection Stx is the main pathogenic factor and necessary for HUS development, loss of the stx gene during the early stage of an enterohemorrhagic *E. coli* O157:H7 and O26 infection has been consistently reported (Bielaszewska et al., 2007; Friedrich et al., 2007) strengthening the evidence that Stx is not needed for *E. coli* persistence and therefore does not induce effective immune evasion.

This work has confirmed previous work reporting that the terminal rectum is the major colonisation site for *E. coli* O157:H7 in cattle (Naylor et al., 2003a). Nevertheless, the main mechanisms driving the terminal rectal tropism and the selective advantage it provides are still unknown. It has been shown that several mutations prevent the expression in *E. coli* O157:H7 of conventional *E. coli* fimbrial clusters that could mediate bacterial adhesion (Low et al., 2006b; Roe et al., 2001; van Diemen et al., 2005). Work with isogenic deletion mutants in cattle has corroborated the importance of AE lesion formation and demonstrated that the only virulence factors that hamper colonisation of the terminal rectum by *E. coli* O157:H7 were TTSS related or the lack of pO157 (Naylor et al., 2005b, Sheng et al., 2006a). One hypothesis that could explain the rectal tropism is cell-to-cell contact, one of the key mechanisms that induces the expression of the type III secretion system (Roe et al., 2003). To test the hypothesis that the colonization of the bovine terminal rectum could

be caused by cell-to-cell contact (bacteria to rectal epithelium) induced by the contraction of the sphincter muscle, a simple surgical procedure is proposed. The procedure to test this hypothesis will be a chemical anal sphincterotomy, using botulinum toxin, in a group of cattle to limit the muscular pressure over the terminal rectum area. Chemical sphincterotomy is less invasive and reduces the residual risk of incontinence when compared with surgical sphincterotomy in humans (McCallion and Gardiner 2001; Minguez et al. 2003). Animals would have to be used because the *in vitro* work supports the claim that mechanical forces induce bacterial adherence to cell cultures, but the validity of this hypothesis, i.e. that the anal sphincter contraction is the cause of the rectal tropism of *E. coli* O157, can only be obtained by proving this physiological mechanism in the living animal. Cattle are a major reservoir of *E. coli* O157 and the main target of intervention strategies aiming to minimise the risk that this bacteria poses to public health. Clarification of the mechanism that induces the initial bacterial adhesion to the host cells will have a significant impact on the design of an effective vaccine. If bacterial adherence was induced mainly by mechanical pressure the efficacy of the vaccine could be better achieved by inducing antibodies against molecules that could impair the fitness of the bacteria instead of targeting putative adhesins.

Our data indicates that *E. coli* O157:H7 colonisation and AE lesion formation induces innate and immune responses in shedding calves. Both findings have been recently corroborated by a bovine-specific cDNA microarray used to characterise gene expression in the bovine terminal rectum mucosa in response to *E. coli* O157:H7 colonization (Li and

Hovde, 2007). The latter study reported significant expression of the NAPDH oxidase and PIGR genes, related with the neutrophil respiratory burst, and the polymeric immunoglobulin A transepithelial transporter, respectively.

The biological relevance of humoral responses is questioned by several authors who report that previous exposure does not reduce shedding (Bretschneider et al., 2007; Hoffman et al., 2006; Johnson et al., 1996; Khaitsa et al., 2003) with re-infection being reported regularly in field and experimental studies (Lahti et al., 2003; Mechie et al., 1997; Shere et al., 2002; Wray et al., 2000), although some authors suggest that prior infection induces partial protection and reduces posterior shedding (Naylor et al., 2007; Sanderson et al., 1999). In general, the evidence is that humoral responses are not an absolute requirement for clearing infection, and that previous exposure does not prevent further re-infections or animals becoming high shedders again. Thus, suggesting that adaptative responses play a limited role in preventing or controlling *E. coli* O157:H7 infection.

Acute intestinal inflammatory responses in the form of a neutrophilic infiltrate were present at the terminal rectum. Marked neutrophilia is also a hallmark of HUS patients and a predictor of outcome, with patients showing high peripheral neutrophil counts at presentation having poor prognosis (Buteau et al., 2000; Fernandez et al., 2000, Roche et al., 2007). It would be interesting to determine if in high shedding animals the neutrophil chemotaxis and microbicidial activity is altered, and if there is a correlation between neutrophil fitness and host

infection susceptibility. This could be achieved by measuring neutrophil function to establish if their phagocytosis activity and oxidative burst capacity is reduced or unchanged, and also if the neutrophils are deactivated and therefore are differentiated towards an inflammatory phenotype such as CD14-reduced and CD16-enhanced membrane expression. It would also be interesting to determine the requirements for complement and antibodies for *E. coli* O157:H7 killing by an opsonophagocytic assay using sera/homogenate from infected cattle to see if restrictions in any of those components restricts bacterial killing. Elucidation of the methods by which neutrophils respond to *E. coli* O157:H7 colonisation will aid in the development of methods to enhance their bactericidial efficiency.

In this work, evidence is presented of mucosal IgA immune responses induced following infection at the bovine terminal rectum and directed against multiple antigens, including TTSS-dependent proteins, O157 lipopolysaccharide, H7 flagellin and OmpC. These isotype-specific antibody responses against *E. coli* O157:H7 secreted, and membrane antigens were detected approximately three weeks after the final serial challenge. Published research to date has examined serum responses: some authors reported a negative correlation between titres and shedding for O157 LPS and H7 IgA (Naylor et al., 2007) and O157 LPS (Johnson et al., 1996; Wray et al., 2000) while other authors report a positive relationship for specific IgGs anti O157 LPS, H7 intimin and a number of TTSS effectors (Bretschneider et al., 2007; Naylor et al., 2007). Although not directly comparable, these studies offer different evaluations of the relevance of the humoral immune responses in protection against *E. coli*

O157:H7 infection. Future work would need to establish the duration of the mucosal IgA titres and their correlation with protection.

This work has also attempted to determine if the external loops (see M&M, sections 2.15, 2.40) of OmpC were recognised as reactive epitopes by the immune sera of calves. Failure to prove the immune reactivity of these sections of *E. coli* O157:H7 OmpC may reflect an inadequate elaboration or selection of the size of antigens, more than their true potential as highly specific immunoreactive antigens of the porin molecule. These strain-specific and most exposed sections of *E. coli* O157:H7 OmpC could provide a viable alternative to the customary, but prone to cross reactions O157 LPS antigen (Chart and Jenkins, 1999; Laegreid et al., 1998; Navarro et al., 2007; Nielsen et al., 2004b) in the development of detection tests. Time constraints prevented the use of alternative techniques of characterisation of immune responses, such as anti OmpC antibody isolation to increase ELISA detection of the peptides or laser scanning confocal microscopy examination.

Finally, it would be interesting to extend a similar work to other small ruminants and in particular the ovine species. Sheep are a known reservoir of *E. coli* O157:H7 and are capable of shedding in amounts exceeding the threshold for histopathological detection of 10^5 CFU g-1 (Cornick et al., 2000; Ogden et al., 2005; Wales et al., 2001a). Their relevance for public health was highlighted by a study in the Grampians that reported sheep as the main source of environmental contamination followed by cattle (Strachan et al., 2005). Further evidence comes from human outbreaks in Scotland associated with sheep faecal

contamination of pasture (Ogden et al., 2002) and water supplies (Licence et al., 2001). Also in Scotland, O157 has been detected in sheep meat and milk (Coia et al., 2001). Sheep meat and cheese is very popular in the Mediterranean countries, South America, and extended areas of Asia and Australia and have reported *E. coli* O157:H7 in their flocks (Blanco et al., 2003; Phillips et al., 2001; Vettorato et al., 2003; Wani et al., 2003) and therefore any advance in the knowledge of *E. coli* O157:H7 infection in small ruminants could have an international impact. Various studies have reported data on numbers of *E. coli* O157:H7 recovered from the alimentary tract orally-challenged animals, including the large bowel and the intrapelvic rectum (Cookson et al., 2002; Cornick et al., 2000 ; Grauke et al., 2002 ; La Ragione et al., 2006; Wales et al., 2001b ; Wales et al., 2001a; Woodward et al., 2003), thus the terminal rectum is recognised in sheep and cattle to be part of the adhesion pattern in the large bowel.

Final considerations

The investigations presented in this thesis have advanced the understanding of *E. coli* O157:H7 bovine infection via exploration of the pathological processes and associated host immune responses. Many new questions and potential areas for further work have opened up during this research, indicating that this important and scientifically interesting topic is still to relinquish all its intricacies.

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Annex

Table I. Daily *E. coli* O157:H7 faecal surface and core CFU g^{-1} counts. Group 1 calves.

		Number of days after experimental challenge																													
Case	F	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
203-R	S	3.E+03	4.E+04			0.E+00	0.E+00	7.E+04	2.E+05	3.E+05			4.E+06	2.E+05	3.E+05																
203-R	C	0.E+00	8.E+02			7.E+02	0.E+00	9.E+02	0.E+00	6.E+02			4.E+03	0.E+00	4.E+03																
204-O	S	3.E+02	2.E+03			3.E+05	5.E+06	7.E+05	7.E+05	2.E+06			6.E+06	0.E+00	5.E+06																
204-O	C	4.E+02	0.E+00			3.E+04	0.E+00	0.E+00	2.E+04	4.E+03			2.E+05	0.E+00	3.E+06																
274-O	S	2.E+03	1.E+02																												
274-O	C	2.E+03	0.E+00																												
276- RU	S	5.E+02	1.E+02			7.E+02	3.E+02	2.E+04	2.E+03	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00											
276- RU	C	6.E+02	0.E+00			0.E+00	0.E+02	8.E+02	0.E+00	3.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00											
332-R	S	5.E+03	4.E+04			6.E+04	4.E+04	4.E+04	2.E+04	4.E+04			2.E+04	2.E+02	2.E+04	2.E+04				3.E+03											
332-R	C	2.E+02	5.E+02			4.E+03	4.E+03	0.E+00	9.E+02	0.E+00			0.E+00	0.E+00	6.E+02	0.E+00				0.E+00											
338-R	S	0.E+00	0.E+00					0.E+00	0.E+00	0.E+00			0.E+00	0.E+00																	
338-R	C	0.E+00	0.E+00				0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00		0.E+00															
449-R	S		1.E+05			6.E+05	6.E+04	3.E+05	2.E+05	0.E+00			4.E+03	4.E+02	5.E+02	7.E+02	3.E+02			5.E+03	0.E+00	0.E+00	2.E+04	7.E+01			2.E+02	0.E+00			
449-R	C		4.E+04			2.E+03	6.E+02	4.E+02	8.E+03	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00			
452-R	S	8.E+04	4.E+05			9.E+04		2.E+05	3.E+04	4.E+04			6.E+03	4.E+03	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00							
452-R	C	2.E+03	1.E+04			4.E+03		2.E+03	7.E+02	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00							
463-O	S	2.E+02	1.E+02			2.E+06	0.E+00	3.E+06	2.E+05	2.E+04			5.E+04	4.E+03	2.E+02	7.E+02	2.E+03			2.E+02	2.E+05	0.E+00	0.E+00	9.E+02			9.E+03	0.E+00			
463-O	C	0.E+00	1.E+02			3.E+05	4.E+05	0.E+00	2.E+03	0.E+00			2.E+02	3.E+02	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00			
473-O	S		2.E+05			6.E+05	3.E+05	0.E+00	0.E+00	0.E+00			2.E+03	5.E+03	0.E+00	4.E+03	2.E+03			2.E+03	1.E+40										
473-O	C		1.E+03			0.E+00	0.E+00	6.E+03	6.E+03	3.E+02			5.E+02	2.E+03	0.E+00	2.E+03	0.E+00			0.E+00	0.E+00										
473-RU	S	4.E+02	0.E+00			0.E+00	0.E+00	0.E+00	8.E+03	2.E+03			0.E+00																		
473-RU	C	6.E+02	1.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00		0.E+00																
480-O	S	4.E+04	3.E+03			0.E+00	5.E+04	9.E+04	2.E+05	3.E+04			4.E+05	0.E+00	5.E+03	2.E+05				0.E+00	3.E+03	2.E+02									
480-O	C	5.E+04	2.E+03			3.E+03	7.E+03	3.E+03	9.E+02	9.E+02			9.E+03	2.E+03	0.E+00	3.E+04				6.E+02	2.E+02	0.E+00									
481-O	S	0.E+00	0.E+00			5.E+03	7.E+03	2.E+04	5.E+03	3.E+04			4.E+03	0.E+00	3.E+03	2.E+03	5.E+03			3.E+04	7.E+04	0.E+00									
481-O	C	3.E+02	3.E+02			7.E+03	8.E+03	2.E+04	6.E+03	8.E+03			0.E+00	0.E+00	4.E+03	2.E+03	5.E+03			8.E+02	9.E+03	3.E+02									
493-R	S	4.E+02	1.E+03				0.E+00	2.E+05	6.E+05	0.E+00			3.E+05	4.E+04		0.E+00	0.E+00			0.E+00	0.E+00										
493-R	C	0.E+00	0.E+00				0.E+00	4.E+04	4.E+04	0.E+00			2.E+03	0.E+00		0.E+00	0.E+00			0.E+00	0.E+00										
507-O	S	5.E+02	2.E+05			3.E+05	4.E+05			7.E+05			5.E+04	2.E+04																	
507-O	C	8.E+02	2.E+05			3.E+06	2.E+05	0.E+00	0.E+00	9.E+04			8.E+03	2.E+04																	
507-O	W							4.E+06	6.E+05																						
540-R	S	2.E+03	4.E+04	0.E+00	2.E+05			2.E+06	3.E+05	3.E+03	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00														
540-R	C	0.E+00	4.E+02	0.E+00	4.E+04			0.E+00	8.E+03	3.E+02	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00														
541-O	S	0.E+00	1.E+04	3.E+05				6.E+04	2.E+04	0.E+00	2.E+04	0.E+00			0.E+00	0.E+00															
541-O	C	0.E+00	6.E+02	4.E+03				0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00															
542-R	S			5.E+03	6.E+03				6.E+05	4.E+05	0.E+00	3.E+03			3.E+03		3.E+00														
542-R	C			0.E+00	8.E+02				9.E+03	0.E+00	0.E+00	7.E+02			4.E+02		0.E+00														
542-R	W	2.E+02	7.E+01					5.E+02																							
551-O	S	0.E+00	8.E+03																												
551-O	C	8.E+04	3.E+03																												
557-R	S	0.E+00	3.E+05			7.E+04	7.E+06	5.E+05	7.E+05	0.E+00			3.E+03	5.E+03	2.E+03	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00									

557-R	C	0.E+00	8.E+03			5.E+02	2.E+04	9.E+03	4.E+03	5.E+03			2.E+02	4.E+02	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00									
566-R	S			2.E+05		4.E+05	3.E+05	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
566-R	C			2.E+05		3.E+02	7.E+03	7.E+03			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
566-R	W				0.E+00																										
566-RU	S	0.E+00	8.E+03			4.E+04	3.E+04	4.E+04	2.E+04	4.E+04			2.E+04	8.E+03	2.E+02	7.E+03	0.E+00			0.E+00	3.E+03	5.E+02	9.E+02								
566-RU	C	2.E+02	8.E+03			2.E+02	2.E+02	2.E+03	7.E+02	8.E+03			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00								
567-R	S	5.E+02	1.E+02			4.E+03	2.E+04	2.E+05	4.E+05	5.E+05			4.E+04	3.E+05	9.E+04	4.E+05				2.E+04	7.E+03	0.E+00	0.E+00								
567-R	C	0.E+00	0.E+00			0.E+00	2.E+03	5.E+02	0.E+00	0.E+00			6.E+02	0.E+00	3.E+03	6.E+03				2.E+02	2.E+02	0.E+00	0.E+00								
574-O	S					7.E+04	4.E+04	3.E+04	3.E+04	3.E+04				0.E+00	5.E+02	6.E+04	0.E+00			0.E+00			0.E+00	1.E+02							
574-O	C					4.E+03	0.E+00	0.E+00	0.E+00	6.E+02				0.E+00	0.E+00	4.E+03	0.E+00			0.E+00			0.E+00	0.E+00							
574-O	W	6.E+02	1.E+02										2.E+02								0.E+00	0.E+00									
576-O	S					0.E+00	0.E+00													0.E+00	0.E+00					0.E+00	0.E+00	0.E+00			
576-O	C					0.E+00	0.E+00													0.E+00	0.E+00					0.E+00	0.E+00	0.E+00			
576-O	W			0.E+00	0.E+00			0.E+00																							
581-O	S				0.E+00	0.E+00	0.E+00	0.E+00										0.E+00	7.E+04	0.E+00	0.E+00					0.E+00	0.E+00				
581-O	C				0.E+00	0.E+00	0.E+00	0.E+00										0.E+00	4.E+02	0.E+00	0.E+00					0.E+00	0.E+00				
583-R	S			5.E+03	2.E+05	7.E+04	4.E+03	5.E+03			6.E+02	2.E+03	0.E+00	2.E+03				0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
583-R	C			0.E+00	4.E+04	0.E+00	2.E+02	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00				0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
586-O	S	0.E+00	0.E+00	2.E+04				9.E+03	0.E+00	3.E+02	2.E+02	0.E+00			0.E+00	0.E+00	0.E+00														
586-O	C	0.E+00	0.E+00	3.E+02				0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00														
587-O	S		1.E+03	4.E+02	0.E+00			5.E+03	2.E+05	0.E+00	2.E+05	4.E+02			0.E+00	0.E+00	0.E+00														
587-O	C		1.E+02	0.E+00	0.E+00			2.E+04	4.E+02	0.E+00	9.E+03	0.E+00			0.E+00	0.E+00	0.E+00														
587-O	W	0.E+00																													
593-R	S	5.E+02	4.E+03	4.E+05	4.E+05			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00					0.E+00														
593-R	C	0.E+00	0.E+00	8.E+03	0.E+00			0.E+00	5.E+02	0.E+00	0.E+00	0.E+00					0.E+00														
593-R	W														0.E+00	0.E+00															
626-O	S	4.E+02	3.E+01			6.E+04	0.E+00	7.E+03	5.E+03	5.E+03			4.E+03	4.E+04	2.E+04	0.E+00	8.E+03			9.E+03	2.E+03										
626-O	C	4.E+02	3.E+01			3.E+03	0.E+00	8.E+02	3.E+02	0.E+00			6.E+02	2.E+03	0.E+00	2.E+03	0.E+00			0.E+00	2.E+02										
677-RU	S	2.E+03	4.E+03			2.E+04	2.E+04	2.E+04	2.E+04	2.E+05			0.E+00	0.E+00	4.E+04	3.E+04	6.E+03			3.E+03	0.E+00		0.E+00								
677-RU	C	0.E+00	1.E+00			0.E+00	7.E+02	4.E+02	8.E+02	2.E+03			0.E+00	0.E+00	4.E+02	3.E+03	6.E+02			0.E+00	0.E+00		0.E+00								
678-RU	S	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00											
678-RU	C	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00											
683-R	S	7.E+02	1.E+04			3.E+06	2.E+07	2.E+05		3.E+05			3.E+04		0.E+00	0.E+00	2.E+04			3.E+04	3.E+04	3.E+00	3.E+04	7.E+03							
683-R	C	0.E+00	2.E+02			3.E+05	5.E+03	2.E+03		7.E+04			2.E+02	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	4.E+03	2.E+03							
683-R	W													5.E+02																	
684-O	S	0.E+00	1.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00				5.E+02		6.E+03	3.E+03			0.E+00	8.E+03	4.E+03									
684-O	C	9.E+02	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00				4.E+02		8.E+02	0.E+00			0.E+00	3.E+02	0.E+00									
684-R	S	6.E+03	4.E+04			0.E+00	4.E+05	0.E+00					0.E+00	0.E+00	0.E+00		0.E+00														
684-R	C	2.E+02	0.E+00			8.E+03	3.E+02	3.E+04					0.E+00	0.E+00	0.E+00		0.E+00														
685-R	S	6.E+03	2.E+04			2.E+04		7.E+04	9.E+03	3.E+02			3.E+03	4.E+04	7.E+02	5.E+03	3.E+04			0.E+00	2.E+03	7.E+03	2.E+04	1.E+02							
685-R	C	2.E+03	7.E+01			0.E+00		4.E+04	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	3.E+02	0.E+00	1.E+02							
686-O	S	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00																	
686-O	C	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00																	
687-R	S		5.E+05			3.E+05	4.E+03	0.E+00		0.E+00			2.E+02	0.E+00	0.E+00	0.E+00				0.E+00											
687-R	C		7.E+04			8.E+02	0.E+00	0.E+00		0.E+00			0.E+00	0.E+00	0.E+00	0.E+00				0.E+00											
690-RU	S	6.E+02	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00															
690-RU	C	8.E+02	0.E+00			0.E+00	0.E+00	0.E+00					0.E+00	0.E+00		0.E+00															

691-O	S	4.E+02	3.E+01				3.E+05	4.E+05	6.E+04	9.E+03			5.E+03		4.E+03	3.E+03	6.E+02			7.E+02											
691-O	C	7.E+02	0.E+00				3.E+04	2.E+04	0.E+00	6.E+02			2.E+02		3.E+03	6.E+03	5.E+02			0.E+00											
699-R	S	0.E+00	1.E+04			8.E+05	8.E+05	8.E+03	0.E+00	3.E+03			9.E+02	4.E+04	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	2.E+03							
699-R	C	0.E+00	3.E+01			5.E+04	3.E+02	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	2.E+03							
705-O	S	0.E+00	0.E+00			8.E+05	6.E+05	2.E+06	8.E+04	8.E+03			3.E+03	4.E+04	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00							
705-O	C	0.E+00	0.E+00			2.E+03	2.E+04	4.E+04	3.E+03	6.E+03			0.E+00	2.E+02	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00							
709-O	S	0.E+00	4.E+04																												
709-O	C	2.E+02	1.E+02																												
710-R	S	6.E+04	8.E+05			8.E+03	0.E+00	0.E+00	2.E+04	0.E+00				0.E+00		6.E+04				7.E+04	3.E+05	7.E+04	3.E+05								
710-R	C	0.E+00	2.E+03			0.E+00	4.E+02	0.E+00	0.E+00	0.E+00				2.E+04	0.E+00	7.E+03				0.E+00	6.E+04	0.E+00	0.E+00								
710-R	W														0.E+00																
713-O	S	0.E+00	4.E+04			0.E+00	5.E+05	3.E+04	4.E+05	0.E+00				7.E+05	6.E+04	3.E+03															
713-O	C	0.E+00	3.E+03			3.E+04	3.E+04	0.E+00	0.E+00	3.E+04			0.E+00	6.E+03	2.E+03	3.E+03															
713-O	W												4.E+04																		
722-R	S	2.E+02				9.E+04	9.E+04	5.E+04	3.E+04	9.E+04			6.E+03	0.E+00	2.E+03	8.E+03	3.E+03			2.E+04	2.E+04	7.E+05	4.E+06								
722-R	C	0.E+00				5.E+02	2.E+03	4.E+03	9.E+02	2.E+03			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	2.E+04								
739-R	S	0.E+00	2.E+02			5.E+03	0.E+00		7.E+03	8.E+02			0.E+00	8.E+02	0.E+00	4.E+04	3.E+03			2.E+04	3.E+04	0.E+00	2.E+04	1.E+02							
739-R	C	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	2.E+02	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00							
739-R	W							0.E+00																							
740-O	S	7.E+03	4.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00																	
740-O	C	8.E+03	1.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00			0.E+00	0.E+00																	
742-R	S	3.E+02	1.E+02			6.E+04	0.E+00	2.E+04	5.E+05	2.E+05					5.E+04	4.E+03	3.E+04			3.E+03	7.E+02		8.E+03	1.E+02			0.E+00	3.E+02	0.E+00	2.E+02	0.E+00
742-R	C	0.E+00	0.E+00			0.E+00	2.E+03	5.E+02	0.E+00	0.E+00			0.E+00	0.E+00	2.E+02	0.E+00	4.E+03			0.E+00	0.E+00	0.E+00	0.E+00	8.E+02			0.E+00	0.E+00	0.E+00	0.E+00	0.E+00
742-R	W												6.E+05	4.E+04																	
824-R	S	2.E+04	5.E+03			2.E+05	3.E+04		0.E+00	2.E+04			2.E+05	8.E+03	5.E+05	2.E+05	0.E+00			0.E+00	2.E+04	4.E+04	5.E+04								
824-R	C	2.E+02	0.E+00			5.E+03	2.E+02		0.E+00	0.E+00			0.E+00	0.E+00	3.E+03	0.E+00	9.E+03			0.E+00	0.E+00	2.E+03	2.E+02								
825-R	S			4.E+03	0.E+00	9.E+04	2.E+04	2.E+05			4.E+04		8.E+03	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
825-R	C			0.E+00	0.E+00	8.E+02	0.E+00	3.E+02			4.E+02		0.E+00	0.E+00	0.E+00			0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
825-R	W											6.E+02																			
826-O	S			2.E+04	2.E+04	3.E+05	3.E+05	2.E+05			5.E+04	6.E+05	5.E+05	4.E+05	3.E+04			5.E+02	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
826-O	C			0.E+00	4.E+02	2.E+04	0.E+00	9.E+02			2.E+03	6.E+05	0.E+00	2.E+03	2.E+03			0.E+00	0.E+00	0.E+00	0.E+00					0.E+00	0.E+00				
868-O	S	2.E+02	7.E+01			5.E+03	5.E+03	2.E+03	2.E+04	2.E+05			3.E+05	3.E+05	3.E+04	6.E+04	2.E+04														
868-O	C	0.E+00	0.E+00			9.E+02	0.E+00	0.E+00	0.E+00	2.E+03			0.E+00	7.E+03	2.E+03	6.E+03	9.E+02														

G = Group, F = Area of faeces samples, S = Faecal surface, C = Faecal Core, W = Whole faeces, R = Rectally challenge, O = Orally chanllenge, Ru = Ruminally challenge

Table II. Descriptive statistics of *E. coli* O157:H7 counts on gastrointestinal tissue washings and contents. Raw data and log10 transformed data, CFU cm^{-1} and CFU g^{-1} counts respectively. Group 1 calves.

	Untransformed data	
	Core	Surface
Number	497	497
Maximum	10,000,000.	16,566,666.7
Minimum	0.	0.
Mean	47,412.9	246,176.7
Trimmed Mean	82,953	2,879
Mode	0.	0.
Median	400.	12,187.8
Standard Error	513,543.6	1,100,516.9
Variance	263,727,050,523.1	1,211,137,441,477.3
First Quartile	1,000	77,500
Third Quartile	6	3,000
Kurtosis	316.0	120.4
	Log transformed data	
	Core	Surface
Maximum	7.0	7.30
Minimum	1.0	0.47
Mean	3.0	3.9
Trimmed Mean	2.9	4.0
Mode	3.3	4.3
Median	2.9	4.0
Standard Error	0.6	0.5
Variance	1.1	1.6
First Quartile	3.3	2.3
Third Quartile	3.8	4.8
Kurtosis	-0.49	0.41

Table III. Group 1 calves examined at *post-mortem*. Gastrointestinal tissue, contents and faecal surface *E. coli* O157:H7 counts obtained on the day of necropsy and expressed as log base CFU cm^{-1} and CFU g^{-1} respectively.

Calf number	Challenge route	Type of sample	Rumen	Bile	Jejunum	Ileum	Ileocaecal valve	Caecum	Proximal Colon 1	Proximal Colon 2	Spiral Colon	Distal Colon	Proximal Rectum	Mid Rectum	Distal Rectum	Recto-anal junction	Faecal core	Faecal surface
244	O	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.				0.	4.5
244	O	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	4.9		
473	O	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	4.2				0.	4.0
473	O	TW	0.		0.	0.	0.	0.		0.	0.	0.	0.	0.	2.6	3.0		
481	O	C	1.8		0.	0.		2.1	2.4		2.2	1.8	1.8	2.3		4.5	2.4	4.1
481	O	TW	0.			0.	1.9		1.5	0.	0.	0.	0.	0.	0.	4.9		
566	RU	C	0.		0.	0.		0.	0.		0.	0.	0.	0.		0.	1.5	3.0
566	RU	TW	0.			0.			0.	0.	0.	0.	0.	0.	0.	1.7		
684	O	C	0.	0.	0.	0.		0.	0.	0.	0.	0.					0.	3.6
684	O	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	3.4		
204	O	C		0.		0.		1.5	2.6	2.1	0.	0.	2.1	4.9			6.6	6.8
204	O	TW	0.7			1.1	3.3	1.9		2.8	1.3	1.3	3.8	4.0	4.0	6.0		
480	O	C	0.		0.	0.		0.	0.		0.	0.	0.	0.		3.2	2.0	2.4
480	O	TW	0.			0.	0.		0.		0.	0.	0.	0.	0.	2.6		
626	O	C	0.		0.	0.		0.	2.7		2.6	2.0	0.	1.5		3.4	2.2	3.4
626	O	TW	0.			0.	2.6			0.	0.	0.	0.	0.	0.	1.2		
691	O	C	0.		0.	0.		0.	2.0		0.	0.				0.	0.	1.8
691	O	TW	0.			0.	0.		0.	0.	0.	0.	0.	0.	1.9	0.		
713	O	C	2.3	0.	0.	0.		3.3	3.2		3.2	3.1	3.3			3.7	3.4	5.0
713	O	TW	0.		0.	0.	1.9	1.3	1.5	1.2	1.1	0.7	0.	0.		4.7		
740	O	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	2.1	3.9			1.8	4.8
740	O	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	3.7	3.7	4.5		
868	O	C	1.5	0.	0.	0.		3.4	3.4	3.2	3.6	3.6	3.4	4.9			3.8	5.0
868	O	TW	0.		1.6	0.	2.9		1.5	1.5	1.7	0.	0.	0.	3.8	5.8		
203	R	C	0.	0.	0.			0.	0.	0.	0.	0.	0.	4.9			3.6	5.5

203	R	TW	0.		0.	0.	0.	0.	0.7	1.0	0.	0.	1.7	4.6	4.6	5.0		
332	R	C	0.		0.	0.		0.	0.		0.	0.	0.	0.		2.4		
332	R	TW	0.			0.	1.2		0.	0.	0.	0.	1.2	0.	1.2	3.7		
567	R	C	0.		0.	0.		0.	0.		0.	0.	0.				0.	1.5
567	R	TW	0.				0.		0.	0.	0.	0.	0.	0.	1.2	2.8		
683	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.	0.		4.2	3.2	3.8
683	R	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	3.4	2.4		
685	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.	2.6		3.5	2.4	5.1
685	R	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	1.9	3.2	4.4		
710	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	1.8				4.4	5.9
710	R	TW	0.			0.	0.7	0.	0.	2.0	0.	0.	0.7	2.3	5.0	6.0		
722	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	4.8				4.3	5.9
722	R	TW	0.					0.	0.7	0.			3.7	4.0	4.0	6.0		
739	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.	0.			0.	4.3
739	R	TW	0.		0.		0.	0.	0.	0.	0.	0.	0.	1.3	1.4	3.9		
824	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.				2.3	4.7
824	R	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	2.6	2.4		
446	R	C	0.	0.	0.	0.		0.	0.	0.	0.	0.	0.	0.		3.6	1.5	4.2
446	R	TW	0.		0.	0.	0.	0.	0.	0.	0.	0.	0.7		1.0	4.4		

CFU counts expressed in log10 O = Oral route R = Rectal route C = Contents TW = Tissue washings

Table IV. Group 1 calves examined immunohistologically for the presence/absence of *E. coli* O157:H7 in post-mortem samples. Tissue bacterial counts expressed in log CFU cm^{-1}.

		Faecal counts (log CFU cm-1)												
Calf number	Challenge route	Rumen	Jejunum	Ileum	Ileocaecal valve	Caecum	Proximal Colon 1	Proximal Colon 2	Spiral Colon	Distal Colon	Proximal Rectum	Mid Rectum	Distal Rectum	Recto-anal junction
481	O	0.0		0.0	1.9		1.5	0.0	0.0	0.0	0.0	0.0	0.0	3.9
M		-		-	-		-	-	-	-	-	-	-	-
566	O	0.0		0.0			0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7
M		-		-			-	-	-	-	-	-	-	-
684	O	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4
M		-	-	-	-	-	-	-	-	-	-	-	-	-
204	O	0.7		1.1	3.3	1.9		2.8	1.3	1.3	3.8	4.0	4.0	6.0
M		-		-	-	-		-	-	-	-	-	-	+
480	O	0.0		0.0	0.0		0.0		0.0	0.0	0.0	0.0	0.0	2.6
M		-		-	-		-		-	-	-	-	-	-
626	O	0.0		0.0	2.6			0.0	0.0	0.0	0.0	0.0	0.0	1.2
M		-		-	-			-	-	-	-	-	-	-
691	O	0.0		0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0
M		-		-	-		-	-	-	-	-	-	-	-
713	O	0.0	0.0	0.0	1.9	1.3	1.5	1.2	1.1	0.7	0.0	0.0		4.7
M		-	-	-	-	-	-	-	-	-	-	-		-
868	O	0.0	1.6	0.0	2.9		1.5	1.5	1.7	0.0	0.0	0.0	3.8	5.8

M		-	-	-	-		-	-	-	-	-	-	-	+
203	R	0.0	0.0	0.0	0.0	0.0	0.7	1.0	0.0	0.0	1.7	4.6	4.6	5.0
M		-	-	-	-	-	-	-	-	-	-	-	-	+
332	R	0.0		0.0	1.2		0.0	0.0	0.0	0.0	1.2	0.0	1.2	3.7
M		-		-	-		-	-	-	-	-	-	-	-
710	R	0.0		0.0	0.7	0.0	0.0	2.0	0.0	0.0	0.7	2.3	5.0	6.0
M		-		-	-	-	-	-	-	-	-	-	-	+
722	R	0.0				0.0	0.7	0.0			3.7	4.0	4.0	6.0
M		-				-	-	-			-	-	-	+

M = Microscopical examination, O = Oral rout, R = Rectal route, - = Negative, + = Positive

Table V. Group 1 calves examined immunohistologically for the presence/absence of *E. coli* O157:H7 in biopsy specimens. Faecal counts expressed in CFUg^{-1}.

Calf number	276	449	473	481	557	583	626	686	699	739	825
MPC	-	-	-	-	-	-	-	-	-	-	-
6FS	3.E+02	6.E+04	3.E+05	7.E+03	7.E+06	4.E+03	0.E+00	0.E+00	8.E+05	0.E+00	2.E+04
M6	-	-	+	-	+	-	-	-	+	-	-
12Fs	0.E+00	4.E+03	2.E+03	4.E+03	3.E+03	0.E+00	4.E+03	0.E+00	9.E+02	0.E+00	8.E+03
M12	-	-	/	-	-	-	-	-	-	-	-

MPC =Microscopical examination of pre-challenge sample M6 = Microscopy 6 days post- challenge, M12 = Microscopy 12 days post-challenge

6FS = Surface faecal sample taken six days post-challenge, 12FS = Surface faecal sample taken twelve days post-challenge

+ = Postive, - = Negative, / = Not obtained

Table VI. Enumeration of granulocytes per 0.25 mm^2 field in exposed group 1 calves and control animals in the terminal and proximal rectum.

Calf number	Terminal rectum										Proximal rectum									
481	3	16	0	6	23	10	2	20	6	2	3	0	0	0	1	0	0	0	0	0
566	12	5	7	16	7	1	15	6	5	4	0	0	0	3	0	3	0	0	0	0
684	4	1	5	8	0	6	12	3	18	1	7	0	1	0	0	13	0	0	2	0
204	5	2	24	6	3	20	2	5	15	7	3	0	0	0	0	2	0	1	2	0
480	12	8	1	4	4	3	4	7	1	16	21	0	0	0	0	3	0	0	0	1
626	3	0	4	4	12	6	4	8	3	5	0	0	0	0	0	0	0	0	3	0
691	3	16	0	6	23	10	2	20	6	2	3	0	0	0	0	7	0	1	0	0
713	12	7	7	5	16	1	15	6	5	4	13	0	0	2	0	3	0	0	0	0
868	4	1	5	8	0	6	12	3	18	1	2	0	1	2	0	11	0	0	0	0
203	3	0	4	4	12	6	4	8	3	5	3	0	0	0	1	0	0	0	0	0
332	12	8	1	4	4	3	4	7	1	16	0	0	0	3	0	3	0	0	0	0
710	5	2	24	6	3	20	2	5	15	7	7	0	1	0	0	0	0	0	2	0
722	18	8	6	4	9	8	2	9	2	4	3	2	2	2	3	2	0	1	2	0
Control 1	5	0	1	1	1	3	0	0	0	1	7	1	0	0	5	25	4	0	0	0
Control 2	2	0	1	0	2	3	0	0	0	0	5	1	0	2	0	1	0	0	0	1
Control 3	2	0	2	1	0	2	0	1	3	7	0	1	0	1	1	0	1	0	1	2
Control 4	1	0	1	1	7	1	0	1	0	14	0	1	0	1	1	0	1	0	1	2
Control 5	0	0	2	1	11	1	0	3	0	12	1	3	0	0	0	1	0	1	1	0

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Nart P, Holden N, McAteer SP, Wang D, Flockhart AF, Naylor SW, Low JC, Gally DL, Huntley JF. 2008. Mucosal antibody responses of colonized cattle to *Escherichia coli* O157-secreted proteins, flagellin, outer membrane proteins and lipopolysaccharide. FEMS Immunol Med Microbiol. Jan;52(1):59-68.

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Escherichia coli O157 : H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the *LEE4* operon

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Enterohaemorrhagic *Escherichia coli* O157 : H7 is a human pathogen that causes no apparent disease in cattle, its primary reservoir host. Recent research has demonstrated that *E. coli* O157 : H7 predominately colonizes the distal few centimetres of the bovine rectum, and in this study, the *LEE4* operon encoding a type III secretion system translocon and associated proteins was shown to be essential for colonization. A deletion mutant of *LEE4* failed to colonize cattle, in contrast to a co-inoculated strain containing a chromosomal complement of the operon, therefore fulfilling 'molecular' Koch's postulates for this virulence determinant. In addition, attaching and effacing (A/E) lesions were detectable in *E. coli* O157 : H7 microcolonies from the terminal rectum of both naturally and experimentally colonized cattle when examined by transmission electron microscopy. This study proves that type III secretion is required for colonization of cattle by *E. coli* O157 : H7, and that A/E lesion formation occurs at the bovine terminal rectum within *E. coli* O157 : H7 microcolonies. The research confirms the value of using type III secreted proteins as vaccine candidates in cattle.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) has emerged in developed countries over the past 20 years as an important cause of human intestinal disease. In addition to bloody diarrhoea, intestinal infection can lead to potentially fatal systemic sequelae resulting from the activity of Shiga toxins. The majority of these infections in the USA, Canada, UK and Japan are caused by *E. coli* O157 : H7 (Nataro & Kaper, 1998). This serotype has been frequently isolated from cattle faeces, and most human *E. coli* O157 : H7 infections originate, either directly or indirectly, from this source (Besser *et al.*, 1999; Borczyk *et al.*, 1987). It is widely acknowledged that controlling *E. coli* O157 : H7 within the bovine population would be an effective method of reducing transmission to humans (Stevens *et al.*, 2002).

Abbreviations: A/E, attaching and effacing; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; Esp, EPEC secreted protein; FAE, follicle associated epithelium; LEE, locus of enterocyte effacement; MPN, most probable number; TEM, transmission electron microscopy; Tir, translocated intimin receptor; TTSS, type III secretion system.

In common with other EHEC and EPEC (enteropathogenic *E. coli*), *E. coli* O157 : H7 contains a pathogenicity island, known as the locus of enterocyte effacement (LEE), that confers the attaching and effacing (A/E) phenotype (Frankel *et al.*, 1998; McDaniel & Kaper, 1997). The LEE encodes a type III secretion system (TTSS) (Hueck, 1998), various translocators and effectors, the outer-membrane protein intimin (Jerse *et al.*, 1990) and its receptor, termed Tir (translocated intimin receptor) (Kenny *et al.*, 1997). The LEE is arranged into several polycistronic operons termed *LEE1* to *LEE5* (Elliott *et al.*, 1998). The *LEE4* operon encodes several proteins essential for the A/E phenotype. These include SepL (Kresse *et al.*, 2000) and EscF (Wilson *et al.*, 2001), both essential components of the LEE TTSS, and Esps (EPEC secreted proteins) A, B, D and F (Knutton *et al.*, 1998; Taylor *et al.*, 1998; Wachter *et al.*, 1999; McNamara & Donnenberg, 1998). EspA forms filamentous extensions to the TTSS with a hollow central channel through which Tir and several other effector proteins are translocated into the host cell via a pore created by EspD and EspB. Amongst the translocated effector molecules are the *LEE4*-encoded EspF, the *LEE5*-encoded Tir, as well as EspG, EspH and Map (Elliott *et al.*, 2001; Tu *et al.*, 2003; Kenny & Jepson, 2000),

and the non-LEE-encoded Cif, EspI/NleA and TccP (Marches *et al.*, 2003; Mundy *et al.*, 2004; Gruenheid *et al.*, 2004; Garmendia *et al.*, 2004). Other than EspF, these are all likely to be expressed in a *LEE4* knock-out strain, but not secreted or translocated into host cells. Deletion of *LEE4* will preclude the formation of A/E lesions, as translocation of Tir into the host cells is prevented. Importantly, intimin expression on the bacterial surface will not be inhibited, thus preserving any alternative functions of this molecule that exist in addition to its interaction with Tir. The alternative functions may include involvement in direct adherence to the intestinal epithelium (Sinclair & O'Brien, 2002).

Recently, it has been demonstrated that the terminal rectum is the primary colonization site for EHEC O157 : H7 in cattle (Naylor *et al.*, 2003; Rice *et al.*, 2003). This has been confirmed in slaughter cattle (Low *et al.*, 2005), and successful colonization can be induced by a rectal swab inoculated with EHEC O157 : H7 (Sheng *et al.*, 2004), although the factors driving the rectal tropism remain unknown. A number of experimental studies on neonatal animals or weaned-fasted animals have demonstrated the formation of A/E lesions in the gastrointestinal tract of animals (Dean-Nystrom *et al.*, 1997, 1999, 2000; Wales *et al.*, 2001), but such lesions have not been shown at the principal colonization site of the terminal rectum in experimentally or naturally colonized cattle. The importance of intimin has been shown for the colonization of cattle by several groups (Cookson & Woodward, 2003; Cornick *et al.*, 2002; Dean-Nystrom *et al.*, 1998), and an extensive series of signature-tagged mutagenesis studies on both EHEC O157 : H7 and EHEC O26 : H^- (Dziva *et al.*, 2004; van Diemen *et al.*, 2005) have identified over 100 genes required for colonization of the bovine intestine, including LEE genes, regulatory genes and surface structures. In these studies, a different model from the current study was used, in which very young calves (1–2 weeks of age) were colonized without the same apparent terminal rectal tropism. In addition, it is not known whether non-O157 EHEC have a tropism for this site. Two of the *E. coli* O157 mutants in LEE genes, *escN* and *map*, were tested in calves alongside the parent strain, and *escN*, but not *map*, was shown to compromise colonization ability. Neither of these mutations was complemented, and recent research has highlighted potentially profound changes in the genome that can occur as a result of the genetic manipulation. Therefore, despite the fact that EHEC type III secreted proteins are being tested as a vaccine to prevent colonization of cattle by EHEC O157 : H7 (Potter *et al.*, 2004), there has not been a definitive study testing a chromosomally complemented deletion of the TTSS or demonstrating A/E lesion formation in normal cattle. The aim of this research was: (1) to characterize the colonization potential in calves and on *in vitro* organ culture of a *LEE4*-deleted *E. coli* O157 : H7 strain compared against the same strain with the *LEE4* operon replaced within the LEE; (2) to determine if A/E lesions are formed by *E. coli* O157 : H7 at the predominant colonization site of both experimentally and naturally colonized cattle.

METHODS

Strains, primers and plasmids. A summary of the strains, primers and plasmids used is provided in Table 1.

Generation of allelic exchange vectors. Primer pairs KO LHS 5′/3′ and KO RHS 5′/3′ were used to amplify flanking regions of

Table 1. Strains, oligonucleotide primers and plasmids used in this study

Strain	Description	
ZAP198	Nalidixic acid resistant, Shiga toxin-negative *E. coli* O157 : H7 (Naylor *et al.*, 2003)	
ZAP984	ZAP198: *LEE4* replaced with kanamycin resistance gene	
ZAP985	ZAP984: kanamycin resistance cassette replaced with cloned *LEE4*	
Primers	**Sequence**	**Construct**
KO LHS 5′	cgagctcccatcctgcaactcctgg	pHY3
KO LHS 3′	cgggatcccattagccattggaaactcac	pHY3
KO RHS 5′	ggatcccttgaaggctaccagggagatattc	pHY3
KO RHS 3′	gactctagaccctcgctgtacgcttaccaa	pHY3
Repair 5′	cgcggatcccatcctgcaactcctggcgtattc	pAJR162
Repair 3′	cgcggatccccctcgctgtacgcttaccaac	pAJR162
Plasmids	**Description**	
pIB307	pMAK705-based vector for allelic exchange; temperature-sensitive replicon (Blomfield *et al.*, 1991)	
pUC4K	Source of kanamycin resistance cassette (Pharmacia)	
pHY3	pIB307 with 5′ and 3′ *LEE4* flanking regions inserted	
pHY10	pHY10 with kanamycin resistance cassette inserted between flanking regions	
pAJR162	pIB307 with cloned *LEE4* inserted	

the *LEE4* operon of *E. coli* O157 : H7 (stx−) ZAP198 (Naylor *et al.*, 2003) under standard conditions. These were cloned into the temperature-sensitive vector pIB307 (Blomfield *et al.*, 1991), creating pHY3. A *sacB-kan* cassette was cloned between the flanking regions at the *Bam*HI restriction site (Roe *et al.*, 2003) to create plasmid pHY10. To allow subsequent 'repair' of any deletion, the *LEE4* operon was amplified using primer pair Repair 5′/3′ by long-range PCR (Roche) and cloned into pIB307 to create construct pAJR162.

Creation of the *LEE4* deletion and complemented strains. The allelic exchange method of Hamilton *et al.* (1989) was used to delete and then repair the entire *LEE4* operon from *E. coli* O157 : H7 (ZAP198), generating strains ZAP984 and ZAP985, respectively. To create ZAP984 (Δ*LEE4*), plasmid pHY10 was electroporated into ZAP198 and transformants selected on Luria–Bertani (LB, Melford Laboratories, UK)/chloramphenicol (C) (30 $\mu g\ ml^{-1}$) agar plates incubated at 30 °C. Transformants were passaged repeatedly in LB/kanamycin (K) (12·5 $\mu g\ ml^{-1}$) broth at 42 °C to obtain co-integrates. Further passage at 30 °C in LB/K followed by replica plating on LB/K and LB/C plates allowed identification of clones in which a secondary recombination event resulted in the *LEE4* operon being replaced by the *sacB-kan* cassette. To generate the complemented strain ZAP985, plasmid pAJR162 was electroporated into ZAP984 and the above procedure repeated with the following differences: primary integrates at 42 °C were selected by C instead of K, the second set of cultures at 30 °C was performed without antimicrobial selection, and the correct clones were identified by sensitivity to both K and C on replica plates.

Validation of genetically engineered strains. Both the *LEE4* deletion mutant and its complemented derivative were compared against the parent strain for their ability to grow in two liquid media. Overnight stationary phase cultures in LB broth were inoculated at a dilution of 1 in 100 into both LB broth and minimal essential medium (MEM)/HEPES (Sigma-Aldrich). Growth curves at 37 °C (aerated) for all strains were shown to be similar by measurement of OD_{600} (1 cm cuvette path length in a Cecil 2021 spectrophotometer). Expression of LEE-encoded proteins was assessed by the labelling of bacteria by indirect immunofluorescence. Samples of mid-exponential-phase MEM/HEPES cultures were fixed in paraformaldehyde (4 %, w/v) and processed for immunofluorescence microscopy using specific EspA and intimin antibodies (Roe *et al.*, 2004). Larger volumes (50 ml) of cultures grown under the same conditions were used to obtain secreted protein preparations for SDS-PAGE followed by colloidal blue staining and Western blotting with anti-EspD antibody (Roe *et al.*, 2003).

***In vitro* adherence of ZAP198 wild-type, the *LEE4* deletion and complemented strains to bovine intestinal epithelium.** ZAP198, 984 and 985 were prepared for inoculation of mucosal explants. Fresh colonies on LB agar plates were used to inoculate 5 ml MEM/HEPES that was incubated with shaking overnight (37 °C). Fresh pre-warmed MEM/HEPES (4 ml) was inoculated with 1 ml of overnight culture and incubated for 3 h (37 °C). All strains were standardized to OD_{600} 0·5 immediately prior to application to tissue explants. Tissue was obtained from two conventionally reared, weaned Holstein/Friesian male calves aged 10 and 12 weeks. Following euthanasia with intravenous pentobarbitone, full-thickness pieces of normal terminal ileum and ileal Peyer's patch were excised and placed into ice-cold MEM/HEPES with minimal delay. After transport to the laboratory, 4 × 4 mm pieces of intestinal mucosa were cut from the underlying muscle layers and placed on individual sterile foam pads. Pre-warmed culture medium [90 % RPMI (Gibco-BRL) and 10 %, v/v, fetal calf serum (Sigma-Aldrich)] was added until the fluid surface was in contact with the tissue edge. A 50 μl portion of standardized bacterial culture for each strain in the mid-exponential growth phase was added to the explant surface of different blocks ($n=4$ for each strain), and the samples were initially flushed with 100 % O_2, sealed, and then incubated in 5 %, v/v, CO_2 for 5 h. Culture medium was changed every 2 h. At the end of the culture period, the samples were washed briefly in fresh culture medium and fixed in 4 %, w/v, paraformaldehyde in PBS (Oxoid). Fixed tissue was embedded in paraffin blocks and routine histological sections prepared. Bacteria on the sections were labelled by indirect immunofluorescence using rabbit anti-O157 antisera (1 in 100) (Mast Diagnostics) and FITC-conjugated goat anti-rabbit secondary antibody (1 in 1000) (Sigma-Aldrich). Tissue was counterstained with propidium iodide (1 $\mu g\ ml^{-1}$) (Sigma-Aldrich) and viewed on a Leica DMLB fluorescence microscope using a 40 × objective. The proportion of fields containing bacteria adhering to intact epithelium was determined blind by assessing 10 sections from each explant. One explant per strain per tissue type per calf was assessed in this way.

Animals and experimental challenge. Experimental calf challenges were performed at the Moredun Research Institute (MRI) in containment level 3 large-animal housing facilities under Home Office licence number 60/3179. Ethical approval was obtained from the MRI Animal Experiments Committee. Calves were reared conventionally on the farm of origin until at least 2 weeks post-weaning and transported to MRI, where they were acclimatised for 1 week prior to challenge. Faecal samples from each calf prior to challenge were confirmed negative for *E. coli* O157 : H7 by immunomagnetic separation (IMS), performed as per manufacturer's instructions (Dynal). At the time of challenge, the mean age of the calves was 19 weeks (range 13–25 weeks). The challenge bacterial strains were grown separately in LB broth (18 h at 37 °C, with aeration) and diluted in sterile PBS to achieve an inoculum of 10^9 c.f.u. of each strain per animal in a total volume of 20 ml. The inoculum was administered to the calves via a stomach tube and washed down with 500 ml sterile PBS (Naylor *et al.*, 2003).

Tissue for microscopy was obtained from separate studies. Two tissue blocks were from calves (aged 3 and 5 months) challenged with the wild-type *E. coli* O157 : H7 strain (ZAP198) administered orally as described above. Post-mortems were performed at 14 and 21 days, respectively, post challenge. The third tissue block was obtained from a naturally colonized 12 month old steer, as described previously (Naylor *et al.*, 2003). Briefly, a known positive herd was identified by field epidemiology conducted by the Scottish Agricultural College. Faecal samples were screened on CT-SMAC plates, and an individual shedding $>10^4$ c.f.u. g^{-1} non-sorbitol-fermenting *E. coli* O157 was selected for post-mortem the following day.

Bacterial enumeration in faeces. Faeces were sampled and separated into surface and core components, as described by Naylor *et al.* (2003). Ten-gram quantities of faeces 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 triplicate onto both sorbitol MacConkey agar plates containing 15 $\mu g\ ml^{-1}$ nalidixic acid (N-SMAC) (Oxoid) and SMAC plates containing 15 μg nalidixic acid ml^{-1} and 12·5 μg kanamycin ml^{-1} (NK-SMAC). All inoculated media were incubated overnight at 37 °C. Non-sorbitol-fermenting colonies were counted, and a colony from each sample tested for O157 LPS using a latex agglutination test kit (Oxoid). The most probable number (MPN) of recovered bacteria was determined as described previously (Naylor *et al.*, 2003). The *LEE4* deletion mutant was enumerated by the MPN obtained from NK-SMAC plates, whereas the complemented strain was enumerated by subtracting the NK-SMAC MPN from the N-SMAC MPN. Enrichment cultures of samples negative by direct culture were established from 1 ml of the neat faecal suspension added to 9 ml of LB containing 15 μg nalidixic acid ml^{-1}. Following 37 °C incubation for 24 h, 100 μl of enrichment culture was spread onto N-SMAC and NK-SMAC plates and incubated overnight at 37 °C.

Electron microscopy. Blocks chosen for processing had $>10^4$ c.f.u. cm^{-2}, correlating with similar levels per gram in faeces. No *E. coli* O157 : H7 microcolonies were detected on tissues from which the levels of *E. coli* O157 were below 1×10^4 cm^{-2}. Blocks were formalin fixed and paraffin-embedded (Wales *et al.*, 2001). Sections (5 μm) were prepared for immunofluorescence analysis using anti-O157 antisera (Mast Diagnostics). From this, the positions of *E. coli* O157 microcolonies within the tissue blocks were determined. In total, three such regions from two animals were then deparaffinized with xylene, rehydrated 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. Seven ultrathin sections (80 nm) were cut from each of the three regions identified above and mounted on copper grids (Agar Scientific). Random regions negative for *E. coli* O157 microcolonies were similarly processed, and no A/E lesions were detectable by transmission electron microscopy (TEM). Specimens were viewed and photographed on a Philips CM12 transmission electron microscope. Tissue blocks from experimentally challenged calves and a naturally colonized steer described previously (Naylor *et al.*, 2003) were processed by this method.

RESULTS

Examination of *LEE4* phenotype in wild-type, deletion and complemented strains

The presence or absence of *LEE4*-encoded proteins in the wild-type and derived strains (ZAP984 and ZAP985) was confirmed by two methods. The first involved direct labelling of EspA filaments on whole bacterial cells using immunofluorescence. Both the parent and the complemented strains produced EspA filaments under culture conditions (MEM/HEPES) known to stimulate expression of *LEE*-encoded factors (Roe *et al.*, 2003). No filaments were observed in the *LEE4*-deleted strain ZAP984 (Fig. 1A–C). In comparison, intimin was detected on the surface of all three strains by indirect immunofluorescence, indicating that the genetic manipulation of *LEE4* did not prevent the production of the adjacent *LEE5* proteins (data not shown).

Secreted proteins from these strains cultured in MEM/HEPES were harvested and examined by SDS-PAGE. Colloidal blue staining of secreted proteins demonstrated a marked reduction in levels produced by the deletion mutant (Fig. 1D). Western blotting for EspD confirmed the absence of this *LEE4*-encoded protein from the deletion mutant and its presence in the supernatant fluids from the wild-type and complemented strains (Fig. 1E). Several other proteins were absent from the secretion profile of the deletion strain but present in the parent and complemented strain preparations.

Bacterial adherence to bovine tissue explants

The wild type and both derived strains were placed on tissue explants of ileal Peyer's patch, containing a high proportion of follicle associated epithelium (FAE), and non-Peyer's patch terminal ileum containing no FAE. The level of adherent bacteria on intact epithelium was determined (Fig. 2). For both tissue types, the level of adherence was significantly decreased for the *LEE4*-deletion and restored to wild-type levels for the complemented strain.

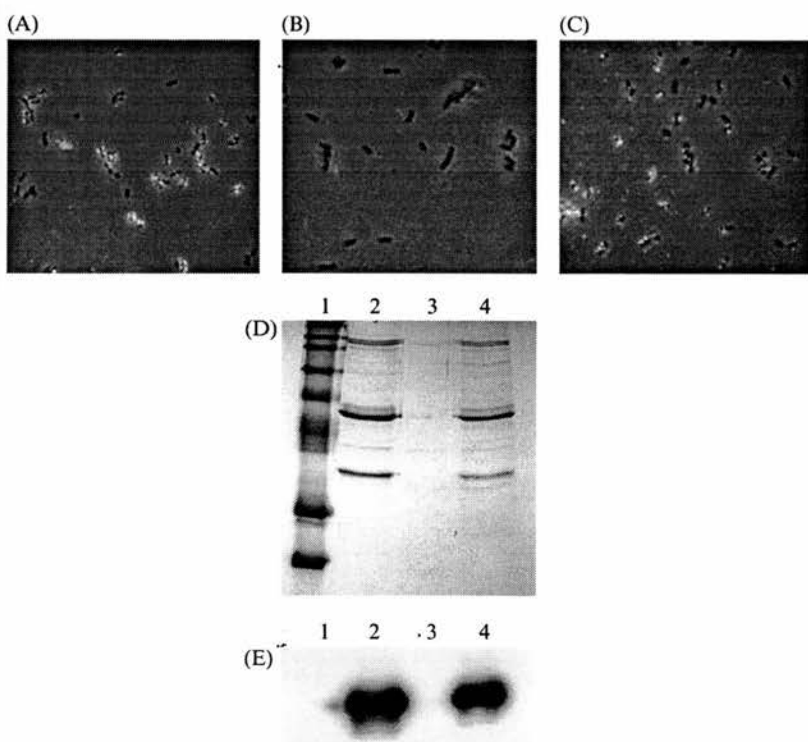

Fig. 1. Phenotypic confirmation of *LEE4* deletion and complementation. Immunofluorescence staining of EspA filaments: (A) wild-type *E. coli* O157 : H7 ZAP198; (B) Δ*LEE4* (ZAP984); (C) Δ*LEE4* complemented with *LEE4* (ZAP985). (D) Colloidal blue staining of supernatant proteins: lane 1, molecular mass marker; lane 2, *E. coli* O157 : H7 ZAP198; lane 3, Δ*LEE4* (ZAP984); lane 4, Δ*LEE4* with *LEE4* reintroduced (ZAP985). (E) Western blotting for EspD in bacterial supernatants; lanes as above.

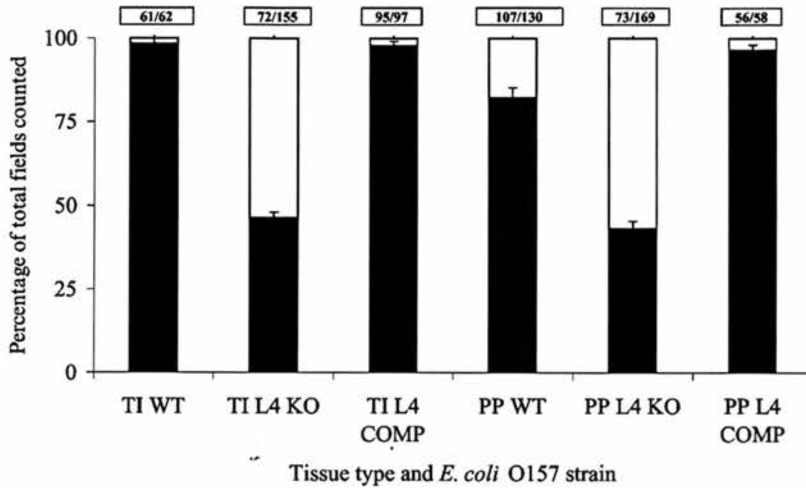

Fig. 2. Adherence of wild-type (ZAP198), Δ*LEE4* (ZAP984) and *LEE4*-complemented (ZAP985) *E. coli* O157 : H7 strains to bovine explant tissues. COMP, complemented; KO, knockout; L4, *LEE4*; PP, Peyer's patch; TI, non-Peyer's patch terminal ileum; WT, wild-type. Assays were carried out as described in Methods. The percentage of fields containing adherent bacteria on intact epithelium is shown by the black bar area. Numbers above the bars show the ratio of positive fields to the total counted. Error bars show the standard error of the between-section mean for each strain ($n=10$).

Experimental colonization of weaned calves

The *LEE4* deletion and complemented strains were simultaneously inoculated orally into six calves, and faecal samples obtained daily until day 14 post challenge. Bacterial counts for both the faecal-surface and core samples were calculated for the two strains. The level of shedding and therefore colonization within an animal using the surface counts (Fig. 3A, B), and the surface : core ratio provide information on terminal rectal-restricted colonization (Fig. 3C). Shedding by the *LEE4*-deleted strain was impaired severely relative to the complemented strain. This strain was not detected from any of the calves beyond day 2 post challenge, indicating a total failure to establish mucosal colonization. In contrast, the complemented strain was consistently shedding beyond day 7 post challenge in four of the six calves. This difference in colonization ability was significant, as determined by Fisher's exact test (one-sided) with a P value of 0·03. In separate calf experiments, the parent strain has been shown to establish persistent infections (S. W. Naylor and others, unpublished results), and shedding of the *LEE4* mutant was clearly reduced when compared to this strain: for example, the same oral challenge dose of the wild-type strain results in faecal shedding ($>10^3$ c.f.u. ml^{-1}) beyond 5 days in 17/22 animals (77 %), compared to 0/6 for the *LEE4* deletion in this study. However, as the experiments were not carried out under identical conditions, the extent of attenuation caused by deletion of *LEE4* cannot be stated conclusively. It is clear however that trans-complementation of the *LEE4* mutant

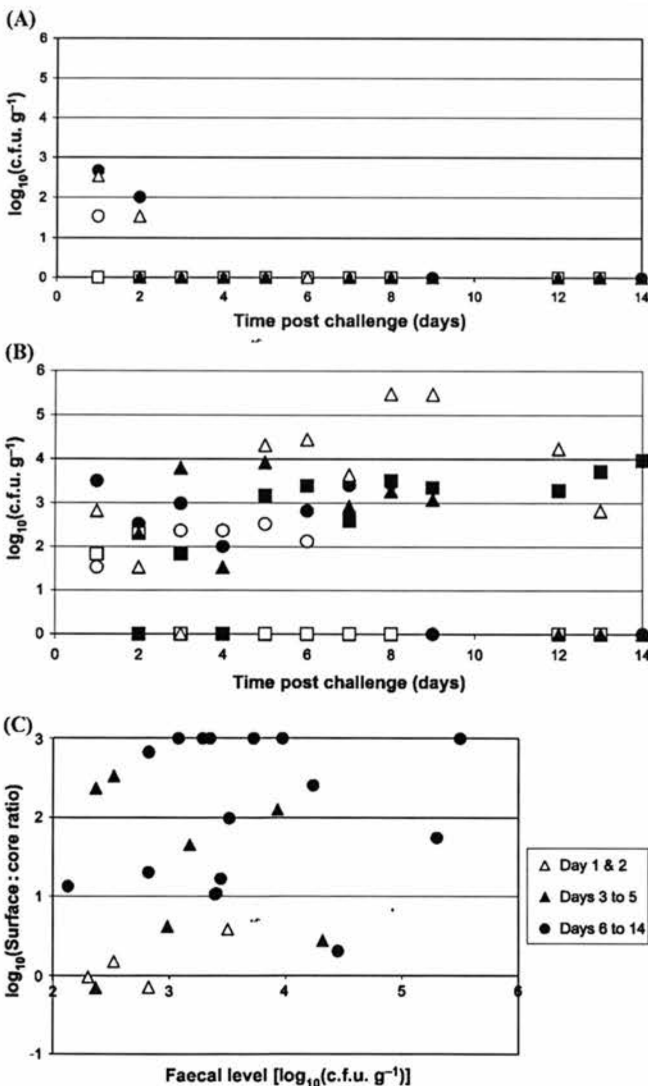

Fig. 3. Experimental colonization of weaned calves. The MPN (c.f.u. g^{-1}) of each strain within surface faecal samples from six calves orally inoculated with (A) the Δ*LEE4 E. coli* O157 : H7 (ZAP984) strain and (B) its *LEE4* chromosomal complement (ZAP985). Individual calves were designated A to F: ○, A; ●, B; □, C; ■, D; △, E; ▲, F. A *y* axis value of 0 represents samples negative by broth enrichment and giving no c.f.u. by direct plating. (C) ZAP985 data with the surface : core ratio plotted against the surface count. This only includes samples in which at least one of the separated components contained 10^2 c.f.u. g^{-1} or greater. Samples with negative core components are assigned a default ratio of 10^3. Three different post-challenge time ranges are indicated.

enhances colonization markedly, confirming a key role for *LEE4* in intestinal colonization.

The temporal pattern of terminal rectal-restricted colonization exhibited by the complemented strain was qualitatively similarly to that observed for the parent strain in our calf colonization model (unpublished data). As indicated in Fig. 3C, the surface : core ratio was low at early time points, but in almost all samples beyond day 6 post challenge, the ratio was greater than 10, indicating that the majority of *E. coli* O157 : H7 within these samples originated from the terminal rectal mucosa (Naylor *et al.*, 2003).

Electron microscopy

Previous research has demonstrated the terminal rectum as the principal site of colonization for *E. coli* O157 : H7 in cattle (Naylor *et al.*, 2003). LEE-mediated A/E lesions have not been demonstrated at this important site in naturally or experimentally colonized cattle. Therefore, TEM was carried out on terminal rectal mucosa obtained from two animals experimentally colonized with the wild-type strain and one naturally colonized steer. Initially, immunofluorescence was used to locate regions containing *E. coli* O157 microcolonies that were reprocessed for electron microscopy. A/E lesions were detected in all positive regions examined ($n=21$) from the three animals (Fig. 4). No attached bacteria were detected in regions negative by immunofluorescence (data not shown). *E. coli* O157 : H7 microcolonies at the terminal rectum comprise bacteria intimately attached to the epithelial surface (Fig. 4). Cells with attached bacteria display the typical features of an effaced brush border and clear pedestal formation below the attached bacteria.

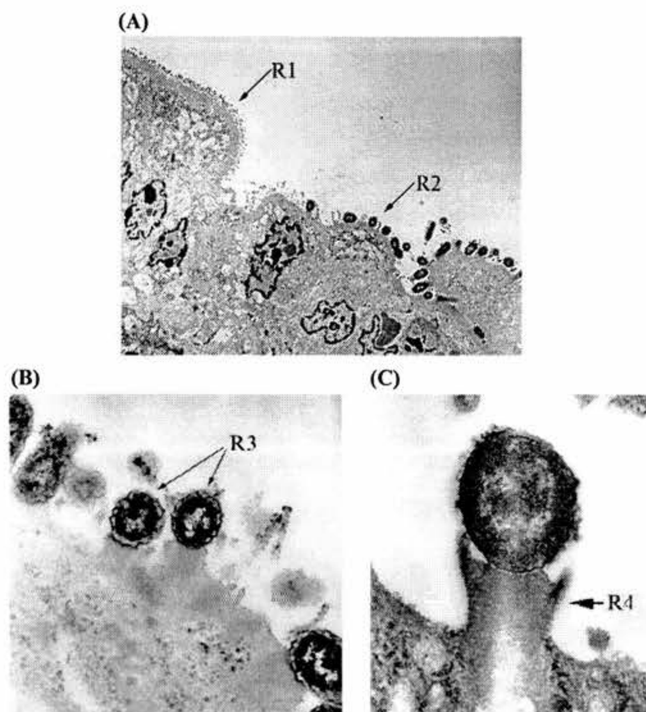

Fig. 4. Transmission electron micrographs of regions containing *E. coli* O157-positive microcolonies. (A) R1 indicates a normal region of mucosa with microvilli. R2 indicates a region with attached bacteria and effaced microvilli on the terminal rectal mucosa of an experimentally colonized calf (×1000). (B) A higher magnification than (A), showing intimately attached bacteria within an *E. coli* O157 microcolony present on the terminal rectal mucosa of an experimentally colonized calf (×5000). (C) A pedestal formed (R4) beneath an attached bacterium within an *E. coli* O157 microcolony on the terminal rectal mucosa of a naturally colonized steer (×10 000).

DISCUSSION

In this study, the role of the *E. coli* O157 : H7 *LEE4* operon in the colonization of cattle is examined by the use of allelic exchange for both deletion and complementation. The data clearly confirm the role of *LEE4* in mediating the colonization of calves in a model that reproduces the restricted colonization of the terminal rectum described previously in both experimentally challenged and naturally colonized cattle (Naylor *et al.*, 2003). A strain lacking *LEE4*, which encodes factors essential for the translocation of Tir and other effector proteins, failed to colonize conventionally reared, weaned calves. When the *LEE4* operon was exchanged back into the deletion strain, this restored the capacity of the strain to secrete effector proteins, produce EspA filaments, adhere to bovine epithelium *in vitro* and colonize cattle at the terminal rectum. As the distribution of a LEE among A/E pathogens has been clearly demonstrated (McDaniel *et al.*, 1995), the present study completes 'molecular' Koch's postulates (Falkow, 2004) for this determinant in the *E. coli* O157 : H7 colonization of the main natural host, cattle.

A common deficiency in many previous animal studies is the lack of any form of molecular complementation. To assess the functional requirement of a micro-organism for genes or groups of genes, the region of interest can be deleted. This manipulation can introduce unknown but significant mutations. To ensure that this has not occurred, it has become established practice to complement the mutation with the region of interest. This is most commonly achieved by transformation with a plasmid including the cloned region. However, this approach has a number of limitations, including plasmid stability and copy number. Therefore, single-copy chromosomal complementation is more desirable, as it can replace the region of interest into its original site. In this study, the whole of *LEE4* was deleted and the complement engineered to precisely replace the operon. To our knowledge, this is the first time that this approach has been used for the assessment of a bacterial colonization determinant *in vitro* and *in vivo*. Moreover, comparison of mutant and complement shedding profiles within the same animals removed between-animal variation in the experiment, and therefore limited the number of study animals required. One possible limitation with the

approach taken in this study is that in order to complement back the wild-type *LEE4* region into the strain containing the deletion and compare direct lineage strains in the experimental colonization study, the deletion strain contained a *sacB-kan*^{r} cassette. This also marked the strain for comparison with the complement. While there is no evidence in the literature that high sucrose levels would be present in the bovine gastrointestinal tract, it is possible that the *sacB* gene could have a negative impact on the strain. Against this, viable bacteria containing the cassette were still detected in the faeces at early time points following oral inoculation, yet no colonization at the terminal rectum occurred (Fig. 3A).

TEM of *E. coli* O157 microcolonies confirmed the presence of A/E lesions for the first time in a naturally colonized animal, and for the first time at the terminal rectum of experimentally colonized animals. A high level of colonization is required to locate microcolonies by this method. The affinity of *E. coli* O157 : H7 for the terminal rectal mucosa results in sufficiently high levels to locate A/E lesions. In addition, lesions were detected within individuals exhibiting sustained colonization, rather than the transient widespread distribution likely to occur soon after a high-dose oral challenge (e.g. Dean-Nystrom *et al.*, 1999). A/E lesions were detected in all regions containing *E. coli* O157 microcolonies and not in regions where *E. coli* O157 was absent. Taken together with the *LEE4* data, these images confirm the importance of type III secretion and A/E lesion formation in the colonization of cattle by *E. coli* O157 : H7. Our findings support the investigation of type III secreted proteins as vaccine candidates to limit colonization of cattle (Potter *et al.*, 2004).

Extensive work by a number of groups has shown the importance of intimin in the colonization of animals by EHEC (Cornick *et al.*, 2002; Dean-Nystrom *et al.*, 1998, 2002; Judge *et al.*, 2004; McKee *et al.*, 1995), but it is unclear from previous work whether its role is limited to Tir interaction or to binding to host cellular receptors, such as nucleolin (Sinclair & O'Brien, 2002). The present study has demonstrated that in the absence of type III secretion, but with intimin expressed, the bacteria are unable to colonize cattle, confirming the pivotal role of the intimin–Tir interaction. By comparison with other studies, the absence of a type III secretion system appears to have a more profound effect on colonization than either intimin (Cornick *et al.*, 2002) or Tir (Stevens *et al.*, 2004) deletions alone. This implies a role for other type III translocated effector proteins in ruminant colonization. While Map has been shown not to be essential for colonization in cattle (Dziva *et al.*, 2004), a number of other secreted proteins have been identified in attaching and effacing pathogens (Kenny *et al.*, 1997; Knutton *et al.*, 1998; Taylor *et al.*, 1998; Wachter *et al.*, 1999; McNamara & Donnenberg, 1998; Elliott *et al.*, 2001; Tu *et al.*, 2003; Kenny & Jepson, 2000; Marches *et al.*, 2003; Mundy *et al.*, 2004; Gruenheid *et al.*, 2004; Garmendia *et al.*, 2004) and some of these may be translocated by *E. coli* O157 : H7 into host cells. Most evidence from cell culture and mouse model systems points to translocated effector proteins modifying host cell functions to favour bacterial persistence in the host: for example, EspF inhibiting apoptosis and disrupting tight junctions (Crane *et al.*, 2001; McNamara & Donnenberg, 1998). These effects may be essential to ensure that any adherent *E. coli* O157 : H7 are able to survive within a hostile niche, regardless of the mechanism of adherence.

All three strains were compared for attachment to ileal explant cultures, and these studies confirmed the importance of *LEE4* for attachment over a 5 h incubation period. While the *LEE4* deletion would need to be tested by direct application to the terminal rectum of cattle to confirm an essential role for type III secretion in the colonization of this site by *E. coli* O157 : H7, the combined colonization, explant and microscopy data presented here do confirm a key role for type III secretion at the bovine terminal rectum. Our ongoing research suggests an increased affinity of *E. coli* O157 : H7 for other FAE-rich sites along the gastrointestinal tract, and we cannot rule out that initial localization at these or other sites increases the likelihood of terminal rectal colonization. An analogous situation has been demonstrated recently for *Citrobacter rodentium* in mice (Wiles *et al.*, 2004). Ileal FAE was used for the explant studies rather than terminal rectal FAE, as the latter cannot be reliably obtained (Mahajan *et al.*, 2005). FAE contains M or M-like cells that sample antigens from the gastrointestinal lumen (Owen, 1999; Nicoletti, 2000). All bacteria in the gut may be sampled by these cells, but certain bacteria, such as *Salmonella*, *Shigella*, EPEC and EHEC may use them to invade or establish colonization (Siebers & Finlay, 1996). The type III secretion system is likely to be a key factor in the interaction of these bacteria with this cell type: for example, inhibiting function to allow colonization or usurping function to allow invasion.

The electron micrographs show classical brush border effacement and degeneration of the cells to which the bacteria are attached, and the host response to and role in this pathology are currently being investigated. Ongoing research into the epidemiology of *E. coli* O157 : H7 and shedding dynamics supports the concept of 'supershedders', a small proportion of *E. coli* O157 : H7-positive animals that contribute the majority of environmental load (Low *et al.*, 2005). These animals are shedding in excess of 10^4 c.f.u. (g faeces)$^{-1}$ and exhibit the terminal rectal colonization previously observed. Specific treatment of this animal subset or vaccination approaches to prevent supershedding, for example using preparations including EHEC type III secreted proteins, are likely to prevent cattle-to-cattle transmission and protect human health.

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RESEARCH ARTICLE

Mucosal antibody responses of colonized cattle to *Escherichia coli* O157-secreted proteins, flagellin, outer membrane proteins and lipopolysaccharide

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mucosal immune responses; *Escherichia coli* O157:H7; flagellin; lipopolysaccharide; secreted proteins; OmpC.

Abstract

The aim of this work was to characterize adaptive mucosal immune responses to *Escherichia coli* O157:H7 at the principal site of colonization in the bovine species. Following experimental infection, extracts from terminal rectum mucosal samples were tested for IgA antibodies by immunoblotting against different bacterial antigens including: whole-cell *E. coli* O157:H7 with and without proteinase treatment, outer membrane and cytoplasmic preparations, secreted protein supernatants and purified *E. coli* O157 lipopolysaccharide and H7 flagellin. Lipopolysaccharide and H7 flagellin preparations were also used to coat enzyme-linked immunosorbent assay plates to determine mucosal IgG1 and IgA antibody titers. In this work, evidence is presented of strong local IgA immune responses induced following infection at the bovine terminal rectal mucosa directed against multiple antigens including type III secretion-dependent proteins, O157 lipopolysaccharide, H7 flagellin and OmpC.

Introduction

Escherichia coli O157:H7 is the most frequently isolated serotype of verocytotoxigenic *E. coli* (VTEC) worldwide (Caprioli *et al.*, 2005). It is associated with serious human disease and infection can result in neurological or renal damage and death (Razzaq, 2006). Human infection is known to induce a vigorous systemic immune response (Li *et al.*, 2000; Currie *et al.*, 2001). Cattle are considered a primary reservoir of these bacteria, and infection may be transmitted to humans through contaminated food, water or by direct animal contact (Beutin, 2006). While *E. coli* O157:H7 is generally considered asymptomatic in the bovine host due to a different distribution of Shiga-like toxin Gb3 receptors in cattle as opposed to humans (Pruimboom-Brees *et al.*, 2000), recent research has identified histopathological damage and lesion formation in the bovine terminal rectal mucosa (Naylor *et al.*, 2005). This is the principal site of *E. coli* O157:H7 colonization in cattle (Naylor *et al.*, 2003; Low *et al.*, 2005; Sheng *et al.*, 2006).

Because of the worldwide human incidence of *E. coli* O157:H7, there is a growing interest in developing strategies that prevent the transmission of this bacteria from the ruminant reservoir. One such approach is in the vaccination of cattle that could prevent or limit bovine infection. Evidence that vaccination may be a viable control option has come from recent studies, in which cattle have been immunized systemically with type III secreted proteins and this partially reduced shedding of *E. coli* O157 following experimental challenge (Potter *et al.*, 2004), although these same authors reconsider this conclusion in a subsequent study (Van Donkersgoed *et al.*, 2005).

It is likely that an effective stimulation of both systemic and local responses will be required and particularly stimulation of IgA responses that play a key role in the immune protection of mucosal surfaces and provides an important line of defense against invading pathogens (Fagarasan & Honjo, 2003; Hamburger *et al.*, 2006). Previous work in cattle has reported serological IgG responses against a variety of antigens following *E. coli* O157 colonization (Cray & Moon, 1995; Pirro *et al.*, 1995; Laegreid *et al.*, 1999; Wray *et al.*, 2000; Potter *et al.*, 2004), and IgA responses against *E. coli* O157:H7 intimin have been detected in nasal

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secretions following nasal inoculation during a vaccine trail (Johnson *et al.*, 1996; Yokomizo *et al.*, 2002). Nevertheless, there is little information on the gastrointestinal mucosal humoral response to *E. coli* O157:H7, in particular following colonisation of the terminal rectum. The objective of this study was therefore to investigate the local mucosal antibody response to *E. coli* O157:H7 following colonization and to define those antigens recognized.

Materials and methods

Bacterial strains and media

The challenge strain was ZAP 198, an *E. coli* O157:H7 isolate from a human patient in Washington State and used in experimental studies previously (Naylor *et al.*, 2003). ZAP 198 has been naturally cured of the Shiga-toxin (Stx2)-carrying bacteriophage and the strain was selected for spontaneous resistance to nalidixic acid to facilitate recovery from feces and tissues. Bacteria for oral inoculation were cultured overnight in a shaking incubator at 37 °C in Luria–Bertani (LB) medium. *Escherichia coli* O157:H7 per gram of feces was enumerated by plating triplicate 10-fold serial dilutions onto sorbitol MacConkey agar plates (SMAC; Oxoid) containing 15 μg – nalidixic acid mL^{-1} (Sigma-Aldrich). To obtain bacterial supernatants containing proteins secreted by type III secretion ZAP 198 was cultured in minimum essential medium (MEM)–HEPES medium as described previously (Roe *et al.*, 2004). *Escherichia coli* AAEC185 (Blattner *et al.*, 1997) is an *E. coli* K-12 derivative that is used routinely as a recipient strain for cloning procedures, in this instance, for the generation of a plasmid used to delete *ompC* from *E. coli* O157:H7 ZAP 198. Cloning steps used LB medium and plates containing the relevant antibiotics: chlormaphenicol (25 μg mL^{-1}) and kanamycin (50 μg mL^{-1}).

Oral challenge of calves

All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and were approved by the local Ethical Review Committee. For this study, eight weaned Holstein-cross calves reared conventionally on a farm and between 8 and 14 weeks old were challenged with *E. coli* O157:H7 isolate ZAP 198 on four consecutive occasions: 1×10^9 (day 0), 1×10^{10} CFU (day 7), 2×10^{10} CFU (day 35) and 2×10^{10} CFU (day 36). Calves were housed together and fed concentrate twice daily and had access to ad lib hay and water. Shedding of *E. coli* O157 in feces was regularly monitored by bacterial culture for a total period of 54 days following the first oral challenge.

Rectal tissue and blood sampling

Serum samples were collected at days – 28, 14, 35 and 49. A total of 32 rectal mucosal samples were taken prechallenge (– 28) and at days 6, 34 postchallenge as well as at postmortem on day 54. On the first three occasions, tissue was excised under local epidural anesthesia achieved by administration of 1 mL of Lidocaine into the intercoccygeal space between C1 and C2. The rectal mucosal samples each weighed between 50 and 75 mg. Excised tissue was mixed with 1 mL of ice-cold phosphate-buffered saline (PBS, pH 7.2) in ribolyzer™ tubes (Hybaid, UK), and then processed in a Hybaid ribolyzer™ for 10 s at 5.5 ms^{-1}. The supernatant of the resultant extracts was removed and centrifuged to remove tissue remnants. The protein content of the supernatant was then determined with the bicinchoninic acid (BCA) protein assay kit (Pierce) according to the manufacturer's instructions. For Western blotting, tissue supernatants averaged 3 mg mL^{-1} of total protein and were used at a 1 : 200 dilution (or 0.013 mg mL^{-1}). For enzyme-linked immunosorbent assays (ELISAs) the supernatants were standardized to a final concentration of 0.5 mg mL^{-1} in PBS containing 0.5 M NaCl and 0.5% Tween 80 (PBS/NaCl/T80). In addition, rectal extracts from three animals with *E. coli* O157-induced histopathological lesions of the rectal mucosa were used as positive controls.

Antigen preparation

Whole-cell *E. coli* O157:H7 (ZAP 198) were harvested from overnight LB broth cultures following two consecutive centrifugation steps (4000 **g** for 5 min). The bacteria were then suspended in PBS. Total protein was measured using a Coomassie Protein Assay Kit (Pierce Biotechnology). Two micrograms of lysates of *E. coli* O157 were loaded per gel lane. The same amounts of crude bacterial preparations were also incubated with 11 μL of proteinase K (25 mg mL^{-1}, specific activity: ≥30U mg^{-1}) (Roche) at 60 °C for 1 h. Bacterial membranes and cytoplasmic proteins were obtained by centrifugation of whole-cell samples (13 000 **g** for 5 min) with the bacterial pellet suspended in sonication buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM phenylmethanesulfonyl fluoride, aprotinin 0.5 μg mL^{-1}) and sonicated on ice twice for 10 s at 15μm with 10-s pauses (Soniprep 150, MSE). This preparation was then centrifuged (13 000 **g** for 15 min at 4 °C) and the supernatant was ultra-centrifuged for 1 h at 50 000 **g** at 4 °C. The pellet was washed twice and resuspended in sonication buffer. The resultant supernatant containing the cytoplasmic proteins was collected and stored at – 20 °C. Bacterial supernatants containing a type III secretion system were generated as described previously (Roe *et al.*, 2004); the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining as published previously (Kenny & Finlay, 1995). An LEE4 mutant of ZAP198 was used as a comparative control (Kenny & Finlay, 1995). Flagellin was purified by the differential ammonium precipitation

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method (Sherman *et al.*, 1988). O157 lipopolysaccharide was obtained from commercial sources (List Biological Laboratories). Mouse monoclonal antibodies against H7 flagellin (Mast Assure) were used to detect H7 antigen by Western blotting as described below.

ELISA

Lipopolysaccharide and H7 flagellin preparations were used to coat ELISA plates to determine mucosal and serological IgG1 and IgA antibody titers. To coat ELISA plates (Thermoelectron 3455), O157 lipopolysaccharide (List Biological Laboratories) was conjugated to polymyxin B as described previously (Currie & Poxton, 1999). Wells were coated with 0.1 μg of H7 or lipopolysaccharide in 0.05 M carbonate buffer, pH 9.6. Blocking was performed with 3% fish gelatin (Sigma G7765) followed by sequential incubation with dilutions of test mucosal biopsy preparations for 1 h at 37 °C in PBS/NaCl/T80, secondary anti-bovine IgA or IgG horse radish peroxidase (HRP) conjugated (Serotec) antibody (1/1000 PBS/NaCl/T80) for 1 h at 37 °C and Sigma Fast OPD was added until color developed (usually 5–20 min depending on the antigen isotype). Between each incubation step, the plates were washed six times with PBS containing 0.5% Tween 20. Finally, the reaction was stopped with 25 μL of 3 M sulfuric acid (Fisher S/9160/PB15), and the OD readings were obtained at 492 nm. Controls consisted of similar dilutions of preinfection mucosal samples, or the omission of the primary test sample.

One-dimensional (1-D) SDS-PAGE and immunoblotting

Proteins were separated using 4–12% NuPAGE Novex Bis-Tris gels (Novex, Invitrogen) with 2-(N-morpholino) ethane sulfonic acid (NuPAGE) MES SDS running buffer under reducing conditions, according to the manufacturer's instructions. Proteins were transferred onto nitrocellulose membranes using a semi-dry transfer apparatus at room temperature. After transfer, the membranes were incubated in PBS/T80/NaCl buffer for 60 min at room temperature to block nonspecific protein binding. The transferred proteins were incubated with the mucosal extracts (0.03 $mg\,g^{-1}$ of mucosal protein). After three washes, the membranes were incubated sequentially with mouse anti-bovine IgA (Dako, 1/500 in PBS/T80/NaCl), biotinylated goat anti-mouse immunoglobulin (Dako 1/2500 in PBS/T80/NaCl) and streptavidin–HRP (Dako, streptavidin–HRP, 1/4000 in PBS/T80/NaCl). The incubation steps were performed for 60 min at room temperature with three washes between each step. Peroxidase activity was revealed by chemiluminescence using an enzyme chemiluminescence (ECL)-reagent (Amersham Life Sciences, Bucks, UK). Control Western blots consisted of incubations with bovine rectal mucosal extracts at the same concentration from: (1) a pool of three animals with histopathological lesions (positive controls) and (2) from unexposed animals (negative control). Western blots in which no primary antibody was added were also carried out as controls.

Two-dimensional (2-D) SDS-PAGE and immunoblotting

Separation of proteins was carried out by isoelectric focusing using the ZOOM IPGRunner System, followed by SDS-PAGE utilizing the NuPAGE Novex 4–12% Bis-Tris ZOOOM gels (Invitrogen) according to the manufacturer's instructions. Two sample loads were used: 170 μL of sample containing 100 μg of crude bacterial protein and 35 μL of sample containing 20 μg of crude bacterial protein. Interfering charged molecules and DNA were removed by submitting the samples to a 2-D Clean-Up kit (Ettan kit, Amersham) according to the manufacturer's instructions. Samples were subsequently resuspended in 155 μL of buffer containing 20 mM dithiothreitol, 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 2% ZOOM carrier Ampholytes, pH 3.5–10 (Invitrogen). Isoelectric focusing (IEF) pH 3–10 strips were rehydrated overnight with the sample buffer and IEF was performed for 4 h at 500 V. Following protein separation by charge, the IEF strips were incubated for 15 min in reducing buffer (NuPAGE, Invitrogen), followed by 15 min in an alkylating solution at room temperature. Two-dimensional separation by protein size was carried out on Bis-Tris Zoom gels. The resultant high-load gel was stained for protein by Simply Blue stain (Novex, Invitrogen) and the low-load gel was submitted to protein transfer and immunoblotting performed as described for 1-D SDS-PAGE gels.

Maldi-ToF-TOF MS

Immunoreactive spots were excised from 2-D gels, destained and digested overnight with porcine trypsin (Promega). The resultant digests were analyzed on a Broker Ultraflex II biospectrometry workstation (Applied Biosystems). The fragmentation data obtained were handled using the MASCOT software package and Mowse scores were calculated. Scores > 74 were indicative of significant protein identity.

Genetic manipulation

The allelic exchange method (Emmerson *et al.*, 2006) was used to replace the *ompC* gene with a *sacB–kan* marker cassette in *E. coli* O157:H7 (ZAP198) to generate strain ZAP 1187. *ompC* flanking regions and the marker cassette were cloned into pIB307 (Blomfield *et al.*, 1991) to create pPN2. The flanking regions were amplified using the

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primer sequences: ACGTGAGCTCATCCGTTGAGTCATCTCAAGC and GCGGATCCTTAACCCTCTGTTATATGCCT (left flanking region) CGGGATCCTCTCGATTGATATCGAACAAAGG and AATTGCATGCCGGTGCAATCACCGTCACG (right flanking region). The *sacB–Kan* cassette was subcloned into the created BamHI site from pKC11 (Holden & Gally, 2004). For the exchange, transformants of pPN3 in ZAP 198 were selected on LB plates containing chloramphenicol (25 $\mu g\,mL^{-1}$) and incubated at 28 °C. Resistant colonies were grown in LBC broth (LB broth containing 25 $\mu g\,mL^{-1}$ of chloramphenicol at 42 °C and subcultured three times to obtain primary integrates. Further subculturing into LBK (LB broth containing 12 $\mu g\,mL^{-1}$ of kanamycin) at 28 °C selected for plasmid excision. *ompC* replacements were screened for by replica plating on LBK and LBC agar plates and confirmed by PCR.

Results

Fecal shedding

Each animal was sampled a total of 38 times during a 50-day period after initial challenge. The counts obtained for each animal are shown in Table 1. All animals had fecal samples with detectable levels of *E. coli* O157:H7 from day 6 and until day 21, with average levels above 10^2. After rechallenges

Table 1. Concentrations of *Escherichia coli* O157 (CFU g^{-1}) on the surface of feces from shedding cattle

				Calves							
CH	SR	BP	DPC	1	2	3	4	5	6	7	8
X	X	X	0								
			1	5433	3133	3033	233	667	67	5367	67
			2	0	200	0	67	33	467	0	100
			3	NC	2000	0	67	0	133	0	400
			4	0	567	0	167	67	600	0	1300
			5	0	8967	0	33	67	133	0	667
X		X	6	0	400	0	100	233	533	0	100
			8	933	19433	100	NC	3533	700	33	0
			9	500	2500	67	0	133	7467	0	0
			10	0	5033	1267	267	5467	21700	67	0
			11	100	0	21233	67	NC	3500	9733	0
			12	0	NC	NC	200	NC	9667	2400	0
			13	0	21933	63367	0	56933	NC	73133	0
	X		14	0	33	10700	167	NC	8233	24900	0
			15	0	0	37733	467	267	NC	8367	0
			16	0	667	50133	33	11100	433	1533	0
			17	0	NC	240667	133	3267	1000	16867	NC
			18	0	67	51433	133	67	0	333	NC
			19	0	133	36600	33	500	167	1067	0
			20	0	300	267	33	100	100	600	0
			21	0	100	2533	0	0	33	600	0
X	X	X	35	0	0	0	0	0	0	0	NC
X			36	533	100	2600	3300	3400	2267	2300	167
			37	167	0	8333	3200	1900	0	1467	233
			38	967	33	867	500	NC	133	1500	0
			39	100	1467	0	633	14633	33	1433	0
			40	3367	33	33	733	200	0	6567	433
			41	3067	33	NC	467	3633	0	533	0
			42	7700	0	0	1133	233	0	7933	0
			43	2333	133	0	0	0	0	1200	0
			44	33	NC	0	0	200	800	767	400
			45		NC	0	67	100	0	100	33
			46	33	67	0	0	0	0	0	100
			47	0	0	0	0	0	0	0	0
			48	67	0	0	0	0	0	133	67
	X		49	0	167	0	0	33	0	0	0
		X	50	0	0	0	0	0	0	0	0

Days 1–50 postinitial challenge (DPC) and cases 1–8. Shedding expressed in CFU g^{-1} of feces.
CH, challenge; BP, biopsy; DPC, days postchallenge; NC, not collected.

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on day 35 and 36, animals shed similar levels of bacteria until day 40. The maximum levels detected in individual animals ranged from 1.3×10^3 to 2.4×10^5 CFU g^{-1} of feces. The average bacterial count for all the samples collected was 3.5×10^3 CFU g^{-1} of feces. Calves did not show any clinical signs during this experiment.

Western blot detection of mucosal IgA antibodies against whole-cell antigens, protein lysates and O157 lipopolysaccharide

Initial analysis of whole *E. coli* O157 responses was performed by 1-D immunoblotting. Depending on the animal, this showed a variable pattern of up to 11 bands of between 38 and 98 kDa that were strongly reactive with IgA when incubated with rectal tissue extracts obtained at 54 days after challenge. For comparison, the prechallenge rectal tissue extracts obtained by biopsy generated a weaker blot with less (< 5) reactive bands (Fig. 1a and b). Proteinase K digestion of the *E. coli* O157:H7 antigen preparation removed the majority of the reacting bands, leaving a diffuse area of recognition at 71–55 kDa for three cases (Fig. 1c). The majority of the extract samples did not show reactivity to lipopolysaccharide, which appeared as a single weak diffuse band migrating below the 70 kDa marker (Western blot not shown).

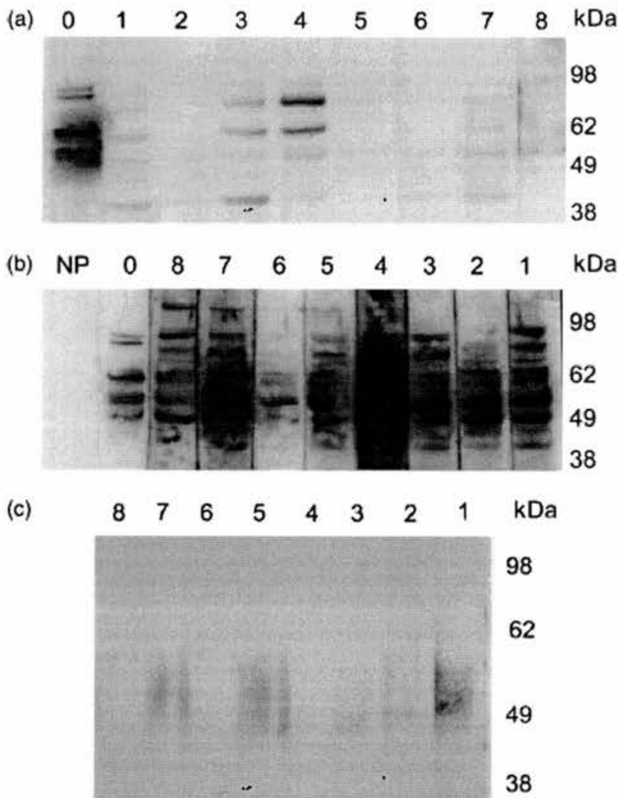

Fig. 1. Reducing SDS-PAGE Western blot of *Escherichia coli* O157 whole-cell preparations showing the IgA-reactive antigens at preinfection (a) and 54 days postinitial infection (b). Blot (c) as for (b), but treated with proteinase K showing diffuse IgA reactivity to areas between 55 and 71 kDa. Each lane was probed with rectal extracts from eight infected calves (1–8). Lane 0 is a positive control, lane NP was as lane 0 but incubated without pooled rectal extract. Protein separation, Western blotting and detection were carried out as described in 'Materials and methods'.

Western blot detection of mucosal IgA antibodies responses to *E. coli* O157-secreted proteins, outer membrane proteins and H7 flagellin

Membrane and cytoplasm protein fractions were analyzed by 1-D immunoblotting. IgA antibody reactivity was predominantly confined to the membrane fractions, although a faint and indistinct reaction against cytoplasmic proteins was observed from the pooled material of highly colonized animals. The reactive membrane proteins ranged from 30 to 90 kDa, with three major bands showing at *c.* 30, 40 and 70 kDa (Fig. 2a). Supernatant proteins from *E. coli* O157:H7 ZAP198 and an *LEE4* deletion mutant (ZAP984) were prepared as described in 'Materials and methods' and used to determine whether there were detectable IgA responses to type III-secreted proteins. Reactive bands with molecular masses expected of EspA (22/25 kDa), EspD-B (37/39 kDa) and Tir (100/110 kDa) (Fig. 2b) were detected when samples were incubated with pooled rectal tissue extracts. No reactive bands were observed when the same extracts were used to blot supernatant proteins precipitated from the *LEE4* mutant. IgA reactivity was also investigated against purified H7 flagellin, and two to three bands were visible between 60 and 200 kDa on extracts from animal 1. A similar band pattern profile was observed following immunoreaction with a commercial monoclonal antibody against H7 antigen; an example from one animal is shown (Fig. 3). All animals were positive (Fig. 5) for this response.

Characterization of a mucosal IgA response to OmpC

Two-dimensional SDS-PAGE, followed by Western blotting, allowed the detection of IgA antibodies against one spot subsequently identified by MS analysis as outer membrane porin C (OmpC) (Fig. 4a). The mucosal samples of all animals showed an IgA response to the OmpC protein. The other two reactive spots were not successfully characterized. A deletion of the gene was then constructed by allelic exchange of ZAP 198 to generate ZAP 1187. The absence of OmpC was confirmed by PCR (data not shown). Comparative immunoblotting of the wild type and *ompC*-deleted strains was then carried out from both 1-D and 2-D SDS-PAGE gels. These confirmed that the originally identified

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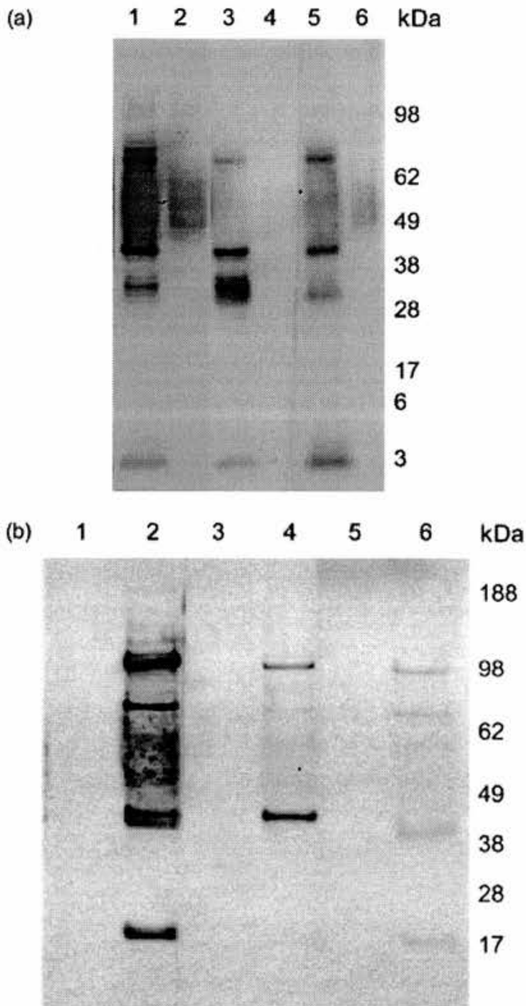

Fig. 2. Reducing SDS-PAGE Western blot of *Escherichia coli* 0157 showing mucosal IgA reactivity to different antigen preparations. For blot (a), outer membrane antigens (lanes 1, 3 and 5) and cytoplasmic proteins (lanes 2, 4 and 6). Blot (b), secreted proteins (lanes 2, 4 and 6) and supernatant of an LEE4 mutant (lanes 1, 3 and 5). Both blots were similarly processed and all lanes were loaded with 1 μg of antigen. For both blots, Lanes 1 and 2 were incubated with a pool of mucosal supernatant from 3 animals with histopathological lesions as positive controls, whereas lanes 3 and 4 were incubated with supernatants from animal 1 and lanes 5 and 6 with supernatants from animal 2. Peroxidase reactions were detected by chemiluminescence although blot (a), lanes 1 and 2 were exposed for a shorter period due to the relatively high intensity of the reaction.

immunogenic protein was no longer detectable in the *ompC* mutant (Fig. 4b and c).

Detection of mucosal antibody responses by ELISA

The mucosal antibody responses measured by ELISA showed an elevated mucosal IgA response at day 54 in all animals against both O157 lipopolysaccharide and H7 flagellin. This response was particularly marked to H7 flagellin when compared with lipopolysaccharide (Fig. 5a and b). Similarly, the readings for IgG antibody were generally higher for H7 than for lipopolysaccharide (Fig. 6a and b). Overall, the rectal mucosal antibody titers increased after repeated *E. coli* O157:H7 challenge.

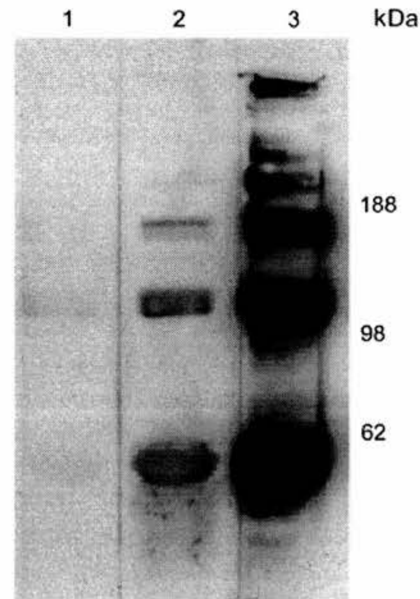

Fig. 3. Reducing SDS-PAGE Western blot showing IgA reactivity to *Escherichia coli* O157 H7-purified flagellin. Lane 1 no primary, lane 2 probed with rectal extracts from case 1 and lane 3 incubated with an anti-H7 monoclonal antibody. Protein separation, Western blotting and detection were carried out as described in 'Materials and methods'.

Discussion

There have been other investigations of the serological immune responses to *E. coli* O157 (Johnson *et al.*, 1996; Lissner *et al.*, 1996; Hoffman *et al.*, 2006) and other EHEC (Widiasih *et al.*, 2004) in cattle. However, this study demonstrates for the first time, that *E. coli* O157:H7 infection in cattle induces local IgA immune responses against membrane and secreted protein antigens at the terminal rectum, the principal site of *E. coli* O157 colonization in the bovine species.

Immunoblotting showed a number of antigens in whole-cell preparations that were recognized by IgA in rectal mucosa extracts collected postchallenge. These bands were more numerous and intensely stained than those detected after a reaction with mucosal extracts from prechallenge tissue, indicating a local IgA antibody response to the repeated *E. coli* O157:H7 challenges. Shared antigens from other *E. coli* present in the normal flora of farm-reared calves, or cross-reactivity with other antigens (Westerman

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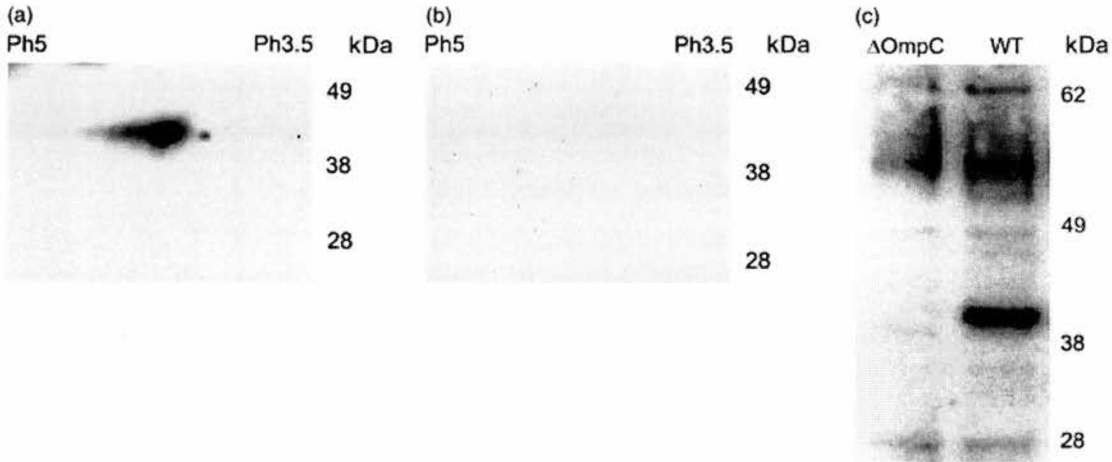

Fig. 4. Reducing 1D and 2-D SDS-PAGE/Western blot analysis of *Escherichia coli* O157. 2-D blot (a) shows the IgA reactive protein OmpC. Immunoblot analysis (b) of the mutant strain (ΔOmpC) indicated the absence of OmpC. Similarly, 1-D SDS immunoblot analysis of outer membrane preparations (c) of the parent wild type (WT) and ΔOmpC strain show in the WT preparation a reactive band on the 42 kDa region absent in the ΔOmpC preparation. Blots were incubated with the pool of mucosal supernatant from three animals with histopathological lesions. Four to twelve per cent Bis-Tris gels, IEF pH 3.5–10. Protein separation, Western blotting and detection were carried out as described in 'Materials and methods'.

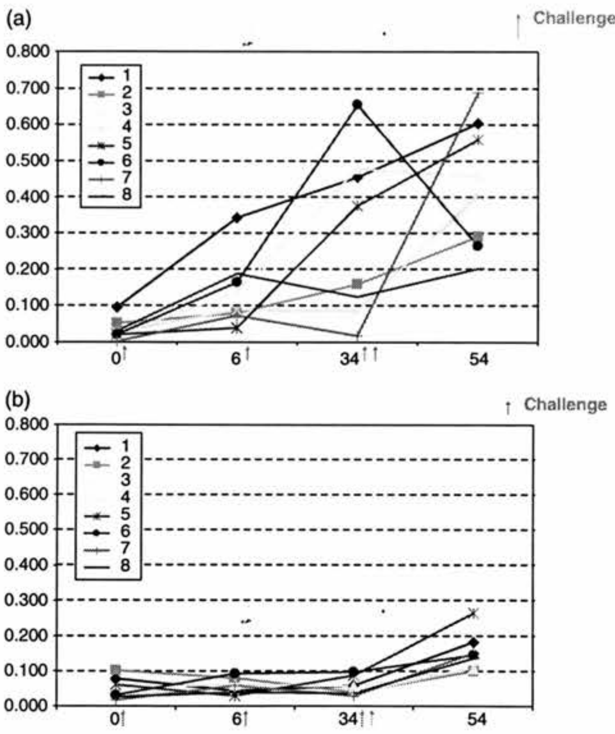

Fig. 5. Levels of rectal mucosa H7-specific IgA (a) and IgG (b) after repeated challenge with *Escherichia coli* O157.

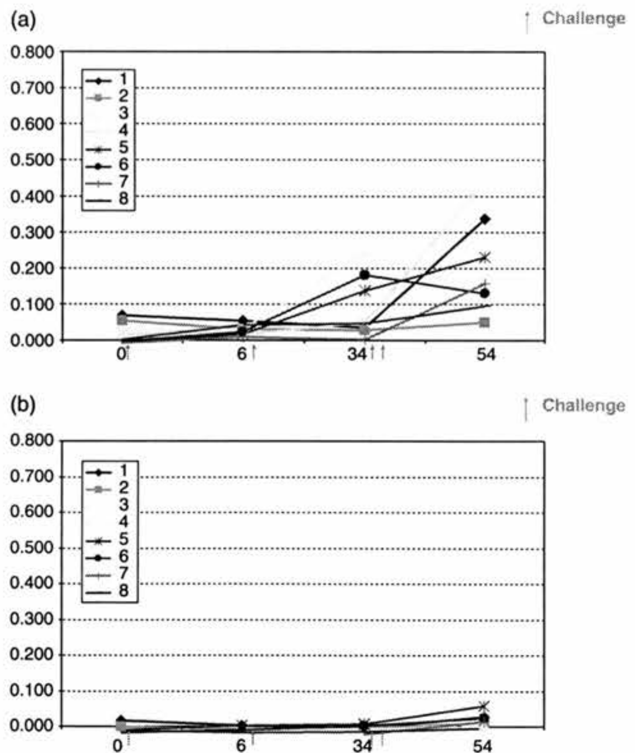

Fig. 6. Levels of rectal mucosa O157-specific IgA (a) and IgG (b) after repeated challenge with *Escherichia coli* O157.

et al., 1997; Stenutz *et al.*, 2006) are likely to explain the low prechallenge reactivity observed.

Proteinase K treatment of whole-cell preparations removed almost all reactive material, suggesting that a high proportion of the antibody targets were protein rather than carbohydrate epitopes. In agreement with this finding, Western immunoblot analysis of purified O157 lipopolysaccharide showed low reactivity. The lower reactivity of the O157 lipopolysaccharide when compared with protein

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antigens was also confirmed by ELISA analyses, carried out with equal antigen loads and primary antibody concentrations. Lipopolysaccharide is one of the main pathogen-associated molecular patterns (PAMPs) used by the mammalian innate immune system to detect Gram-negative bacteria (Backhed *et al.*, 2003; Trent *et al.*, 2006), although pure lipopolysaccharide is considered to be a poor immunogen (Poxton, 1995). Thus, in this case lipopolysaccharide does not appear to be a dominant antigen for the development of a mucosal response.

The rectal mucosal extracts labeled three major reactive bands in the outer membrane preparations of *E. coli* O157:H7, while the cytoplasmic preparations reacted weakly, suggesting that the major immunogenic proteins of the bacterium are surface-exposed. It is possible that like most of the PAMPs that interact with the innate immune system (Blasi *et al.*, 2005; Rezaei, 2006), the main virulence determinants from non invasive bacterial pathogens may be extracellular virulence factors (Parsot, 2005) or membrane components (Kagnoff, 2006). A recent analysis of an expression library recognized by IgG antibodies from immune calves (Kudva *et al.*, 2006) detected predominantly cytoplasmic antigens. However, the work did not identify type III secreted proteins or Shiga toxin against which specific IgG have been reported previously in cattle (Johnson *et al.*, 1996; Yokomizo *et al.*, 2002; Potter *et al.*, 2004). The fact that membrane proteins were the main antigens detected in this study could be a result of restriction site limitations (Rollins *et al.*, 2005) and/or the different tests used, as well as the differences between antibody isotype and local vs. systemic responses, could explain the greater relevance given in this work to membrane protein antigens.

In the present study, culture supernatants containing type III secreted proteins were highly antigenic and reacted strongly with IgA antibodies from infected animals. The proteins recognized were exported by the type III secretion system as they were not detected in an LEE4 mutant supernatant and had molecular masses that correlate with: (1) the needle elongation filament protein, EspA; (2) the pore-generating protein complex, EspB/D and (3) the translocated intimin receptor (Tir). These are all key proteins required for induction of attaching and effacing lesions by *E. coli* O157 and are exposed to the host immune system in the early stages of colonization (Cornelis, 2006). Serological IgG responses against similar proteins have been reported during bovine vaccine trails following vaccination with type III secretion system proteins (Potter *et al.*, 2004) and in human infection (Li *et al.*, 2000). Recent research has found that these proteins are essential for *E. coli* O157:H7 colonization of the terminal rectum (Naylor *et al.*, 2005).

H7 flagellin also elicited strong IgA immune responses, with ELISA values higher than lipopolysaccharide for equal concentrations of antigen and primary antibodies. Unlike lipopolysaccharide, flagellin may activate both innate and acquired immune systems, through the activation of Toll-like receptor 5 and the recruitment of dendritic cells (Yang *et al.*, 1997; Honko & Mizel, 2005; Rumbo *et al.*, 2006). This immundominance of flagellin has also been reported in experimental murine colitis and Crohn's disease patients for IgG (Lodes *et al.*, 2004; Duck *et al.*, 2007) and IgA (Sitaraman *et al.*, 2005). Moreover, *in vitro* studies have found that flagellin is the main inducer of the neutrophil chemoattractant interleukin 8, through the activation of intestinal epithelial cell NF-κβ and mitogen-activated protein kinase pathways (Berin *et al.*, 2002), and *E. coli* O157:H7 infection in cattle is characterized by the induction in the rectal mucosa of neutrophil infiltrates. In contrast to *E. coli* O157:H7 infection in cattle, H7 flagella antibodies have not been detected in the sera of human patients (Chart *et al.*, 1989; Jenkins & Chart, 1999), which may indicate the lack of flagella expression during human infection.

Following 1-D SDS-PAGE/Western blot analysis, further attempts to define the reactive antigens were made by 2-D SDS-PAGE/Western blotting. MS analysis identified OmpC as a bacterial membrane antigen detected by mucosal IgA from all positive animals. The immuno-dominance of this protein has been demonstrated for patients with Crohn's disease where *E. coli* OmpC has been proposed as a reliable marker to characterize infection (Spivak *et al.*, 2006). OmpC elicited higher and prolonged responses that the other major outer membrane protein OmpF, following infection with *Salmonella enterica* (Secundino *et al.*, 2006). The marked difference observed in the number of antigens detected by 1-D and 2-D Western blotting may be due to differences in sensitivity, denaturalization (Harper *et al.*, 1990) or to the absence in the 2-D gels of some antigens (Madden *et al.*, 2002).

In summary, the present study has shown that *E. coli* O157:H7 colonization induces a strong local mucosal antibody response, which is directed at several membrane-associated and secreted proteins. Because IgA has been selected through evolution as the main protective element of the mucosal humoral immune response (Matsunaga & Rahman, 1998), the antigens detected may represent important vaccine candidates. However, further work is required to determine their relevance in protection.

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Responses of Cattle to Gastrointestinal Colonization by *Escherichia coli* O157:H7[▽]

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Recent research has established that the terminal rectum is the predominant colonization site of enterohemorrhagic *Escherichia coli* O157:H7 in cattle. The main aim of the present work was to investigate pathological changes and associated immune responses at this site in animals colonized with *E. coli* O157:H7. Tissue and gastrointestinal samples from a total of 22 weaned Holstein-cross calves challenged with *E. coli* O157:H7 were analyzed for bacterial colonization and pathology. Five unexposed age-matched calves were used as comparative negative controls. *E. coli* O157:H7 bacteria induced histopathological alterations of the rectal mucosa with enterocyte remodeling. This was often associated with removal of the colonized epithelial layer. Immunogold labeling and transmission electron microscopy (TEM) showed *E. coli* O157 bacteria on pedestals, as part of attaching and effacing lesions. These pathological changes induced a local infiltration of neutrophils that was quantified as larger in infected animals. Rectal mucosal immunoglobulin A responses were detected against the *E. coli* O157:H7 antigen. This work presents evidence that *E. coli* O157:H7 is not a commensal bacteria in the bovine host and that the mucosal damage produced by *E. coli* O157:H7 colonization of the terminal rectum induces a quantifiable innate immune response and production of specific mucosal antibodies.

Enterohemorrhagic *Escherichia coli* (EHEC) infection has emerged in the last 20 years as a cause of diarrhea that can lead to the more serious consequence of hemolytic-uremic syndrome and thrombotic microangiopathy. The majority of EHEC infections are caused by *E. coli* O157:H7 (24), and this serotype has been isolated frequently from cattle feces. Many human EHEC O157 infections originate, either directly or indirectly, from exposure to cattle feces (17), and a key step in protecting humans from EHEC infection is to understand and control *E. coli* O157:H7 colonization of cattle.

Experimental challenges have suggested a variety of colonization sites in cattle (4, 5, 12). However, more recently, the terminal rectal mucosa has been identified as the major site of *E. coli* O157:H7 colonization (25), and this finding has been confirmed in slaughter animals (20). From an understanding of where *E. coli* O157:H7 colonizes the bovine intestinal tract, there is an opportunity to examine pathological changes at the site and to determine whether these changes correlate with the development of immunological responses. The main aim of the research is to underpin methods to control this pathogen in its main animal reservoir.

A feature of *E. coli* O157:H7 infection is the formation of attaching and effacing (A/E) lesions, characterized by the elimination of the microvilli and intimate enterocyte attachment (7, 16). In vivo, A/E lesions are present at the terminal rectum of naturally and experimentally infected cattle, and inactivation of the type III secretion apparatus that is essential for this phenotype prevents *E. coli* O157:H7 colonization of cattle (27). The profound alteration of enterocyte morphology associated with A/E lesions has also been reported to be accompanied by an increase in neutrophils and eosinophils in the lamina propria of the large intestine (37), the colon and cecum (6), the gall bladder (35), and sections of ligated ileal loops (32). This type of inflammatory reaction has been described in the intestinal tract exclusively for experimental infections of gnotobiotic, neonatal, or immunosuppressed calves and in sites other than the terminal rectum. However, to date, the response to colonization at the principal colonization site has not been investigated.

Identification of the terminal rectum as the tissue targeted by *E. coli* O157:H7 in cattle allows the study of the pathological changes and associated innate and adaptive mucosal responses. Thus, this study had two objectives: first, to determine if *E. coli* O157:H7 colonization of the bovine terminal rectum induces pathological changes, in terms of both ultrastructural change to the mucosal epithelium and evidence of inflammation, and second, to investigate local immune responses to colonization. Additionally, this work aimed to confirm in a larger number of animals the previously reported findings of *E. coli* O157:H7 tropism for the terminal rectum.

MATERIALS AND METHODS

Animals. Fifty-four weaned Holstein-cross calves were reared conventionally on a farm before transfer to Moredun Research Institute for the experimental

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procedures (authorized by Home Office license 60/3179). The animals were between 8 and 14 weeks of age on arrival and were then penned individually for the study. Calves were fed concentrate twice daily and had access ad libitum to hay and water. Feed, water, and bedding were provided separately for each animal to minimize cross-contamination, and each was haltered to reduce the opportunity for fecal-oral transmission. Five unexposed calves of similar age, breed, and background were used as controls for the histopathological studies, and a separate group of four calves was challenged specifically for ultrastructural investigations of the colonized rectal mucosae.

Bacterial strains. The challenge strain of *E. coli* O157:H7 was ZAP 198 isolated from a human patient in Washington state and was used previously in experimental studies (25). ZAP 198 has been naturally cured of the verocytotoxin-carrying bacteriophage, and the strain was selected for spontaneous resistance to nalidixic acid to facilitate recovery from feces and tissues. ZAP 198 possess genes for enterohemolysin, intimin-γ, EspA, and EspB. For the preliminary examination of adaptive responses, whole-cell extracts of *E. coli* K-12 MG1655 (2) and *E. coli* O26 ZAP 1082 (28) were used as controls in Western blots.

Calf colonization. Fecal samples were taken at least twice from each calf, prior to experimental challenge, and were confirmed negative for *E. coli* O157:H7 by immunomagnetic separation. The challenge *E. coli* O157:H7 strain was grown overnight in Luria-Bertani (LB) broth at 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 oral inoculum was administered to the calves via stomach tube and washed down with 500 ml of sterile PBS or by direct administration through a cannula to the rumen in six calves. Initial analysis showed that the ruminal inoculation and the challenge via nasogastric tube did not differ in terms of efficacy, and the former method was halted. Therefore, for analytical purposes the ruminally challenged animals are included within the orally challenged group. Rectal challenge was carried out by loading a large cotton swab with the challenge inoculum, followed by direct application to the mucosal surface of the recto-anal junction (RAJ). In total, 24 calves were challenged via the rectal route and 30 by oral or ruminal administration.

Postmortem examinations were carried out with 22 animals shedding detectable levels of *E. coli* O157:H7 bacteria beyond day 14 postchallenge. Ten of these animals had been experimentally challenged with *E. coli* O157:H7 by direct rectal application and 12 by oral administration as described recently (26). Tissue samples were collected from the gastrointestinal tract for bacterial counts and histopathology. Tissue samples were taken from the rumen, jejunum, Peyer's patch, cecum, ileocecocolic valve lymphoid patch, proximal colon, proximal colon lymphoid patch, spiral colon, and distal colon and at the proximal, mid, and distal rectum (at 20, 10, and 5 cm proximal to the RAJ) and from the RAJ itself. Luminal contents were collected from the same sites for bacterial culture, together with bile from the gall bladder.

Rectal biopsy samples were taken from 11 animals, under local epidural anesthesia, at prechallenge and on two further occasions at 6 and 11 days after challenge. Local anesthesia was achieved by epidural administration of 1 ml of lidocaine into the intercoccygeal space between C1 and C2. The biopsies consisted of pieces of rectal mucosa weighing between 50 and 75 mg that were excised from the terminal rectum.

Isolation of *E. coli* O157:H7 and enumeration. Feces were caught upon defecation and separated into core and surface components. The concentrations of *E. coli* O157:H7 bacteria in feces, intestinal contents, and tissues were estimated as described previously (25).

Histopathology. Tissues taken at postmortem examination for histopathology were immediately fixed in 4% paraformaldehyde. Thirteen animals were selected for histopathological examination. Cell counts were made of total granulocytes, eosinophils, neutrophils (total granulocytes minus eosinophils), mast cells, and γδ T cells from the lamina propria of the rectum and large intestine. Total granulocyte numbers were obtained from sections stained with hematoxylin and eosin, mast cells from sections stained with toluidine blue (0.5% in 0.5 N HCl [pH 0.5]) for 1 h (9), and eosinophils from sections stained with carbol chromotrope solution for 1 h and counterstained for 10 s with hematoxylin (18). IL A29 was used to detect the γδ T-cell marker BoWC1 (15) in immunostained sections.

Paraffin sections (5 μm) of paraformaldehyde-fixed tissues were used to detect *E. coli* O157:H7 by the horseradish peroxidase (HRP)-streptavidin method. The primary antibody was rabbit anti-O157 polyclonal antibody (1:100 for 60 min at room temperature; Mast-Assure), followed by HRP-streptavidin-conjugated mouse anti-rabbit monoclonal antibodies (Sigma-Aldrich). In some studies, paraformaldehyde-fixed tissues were used to detect *E. coli* O157 by immunofluorescent antibody labeling. These cells were processed as described above, except that the secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma-Aldrich).

Cell counts. These were performed for eosinophils, neutrophils, mast cells, and γδ T cells from the lamina propria of the rectum and large intestine. Using a power calculation of 0.8 and 95% significance level, we counted five fields and two different areas for each of the sections. Cell counts were expressed as cells per 0.25 mm^2. Bacterial counts were obtained after counting 10 colonies in two different RAJ tissues from each of five animals.

Electron microscopy. Two different techniques were used to identify *E. coli* O157 in ultrastructural studies. From the group of thirteen animals selected for histological examination, areas of three tissue sections from two animals where *E. coli* O157 had been identified by immunofluorescent antibody were circumscribed, and the tissue in the matching areas of the paraffin blocks were precisely excised and reprocessed for transmission electron microscopy (TEM). From the group of four calves challenged specifically for electron microscopy studies, tissues were fixed in 2% formaldehyde and 0.1% glutaraldehyde, and bacteria were labeled with the same primary antibody used for light microscopy, with a secondary goat anti-rabbit immunoglobulin G (IgG) 10-μm gold-labeled antibody (1:500 for 60 min at room temperature; British Biocell). Images were processed with PaintShop Pro (Jasc Software).

Determination of mucosal antibody responses. Rectal mucosa (weighing approximately 2 g) samples were excised at postmortem examination from nine animals and stored at −70°C. After being thawed, 50 to 75 mg of the biopsy sample or rectal mucosal tissue was mixed with 1 ml of ice-cold PBS (pH 7.2) in RiboLyzer tubes (Hybaid, United Kingdom) and then processed in a Hybaid RiboLyzer for 10 s at 5.5 ms^{-1}. The supernatant of resultant homogenate was removed and centrifuged to remove the tissue. The protein content of the supernatant was then determined with the bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's instructions and standardized to a final concentration of 5 mg $gram^{-1}$. Whole-cell samples of *E. coli* O157:H7, *E. coli* O26, and *E. coli* K-12 were prepared from overnight LB broth cultures, and heated for 10 min at 70°C in loading buffer, 5 μl of sodium dodecyl sulfate, and 2 μl of reducing agent (Invitrogen), and each lane was loaded with 10 μg of bacterial protein and separated using 4% to 12% NuPAGE Novex bis-Tris gels (Novex, Invitrogen) with NuPAGE morpholineethanesulfonic acid-sodium dodecyl sulfate running buffer. Lysates of *E. coli* O157:H7 were made by trypsinization of whole-cell samples for 45 min at 70°C with sequencing-grade-modified trypsin (Promega). Gels were transferred onto nitrocellulose membranes, using a semidry transfer apparatus at room temperature. After being transferred, the membranes were incubated in a PBS-T80-NaCl buffer consisting of PBS, 0.5% Tween 80, and 0.5 M NaCl for 60 min at room temperature to block nonspecific protein binding. The transferred proteins were incubated with the supernatant from the mucosal homogenates (0.03 mg $gram^{-1}$ of mucosal protein). Following three washes, the membranes were incubated sequentially with mouse anti-bovine IgA (1/500 in PBS-T80-NaCl; Dako), biotinylated goat anti-mouse Ig (1/2500 in PBS-T80-NaCl; Dako), and streptavidin-HRP (streptavidin-HRP, 1/4,000 in PBS-T80-NaCl; Dako). The incubation steps were performed for 60 min at room temperature with three washes between each step. Peroxidase activity was revealed by chemiluminescence using ECL (Amersham Life Sciences, Bucks, United Kingdom) reagent. Control Western blots consisted of incubations of bovine rectal homogenate at the same concentration from unexposed animals and Western blots in which no primary antibody was added.

Statistical analyses. *E. coli* O157:H7 cell counts within fecal and tissue samples were calculated by determining the mean plate count at the most relevant dilution for each sample and by multiplying the dilution factor to convert to CFU g^{-1} (feces or gastrointestinal tract contents) or to CFU cm^{-2} (mucosal samples). The concentration (CFU g^{-1} plus 1) was log_{10} transformed. Student's *t* test was used to compare means of samples, with a paired *t* test used where a natural pair existed. The chi-square test was used to analyze proportions. Where the number of observations was low in some categories, Fisher's exact test was used. The kappa test was used to qualify the level of agreement, where the null hypothesis is that there is no more agreement than might occur by random chance. Data were processed using Excel (Microsoft) and Minitab (Minitab Inc.) software.

RESULTS

Site of colonization. Out of a total of 54 animals, 46 became colonized for longer than 5 days, and a postmortem examination was carried out on 22 animals still shedding detectable levels of *E. coli* O157:H7 bacteria beyond day 14 postchallenge. Ten of these animals had been challenged by direct rectal

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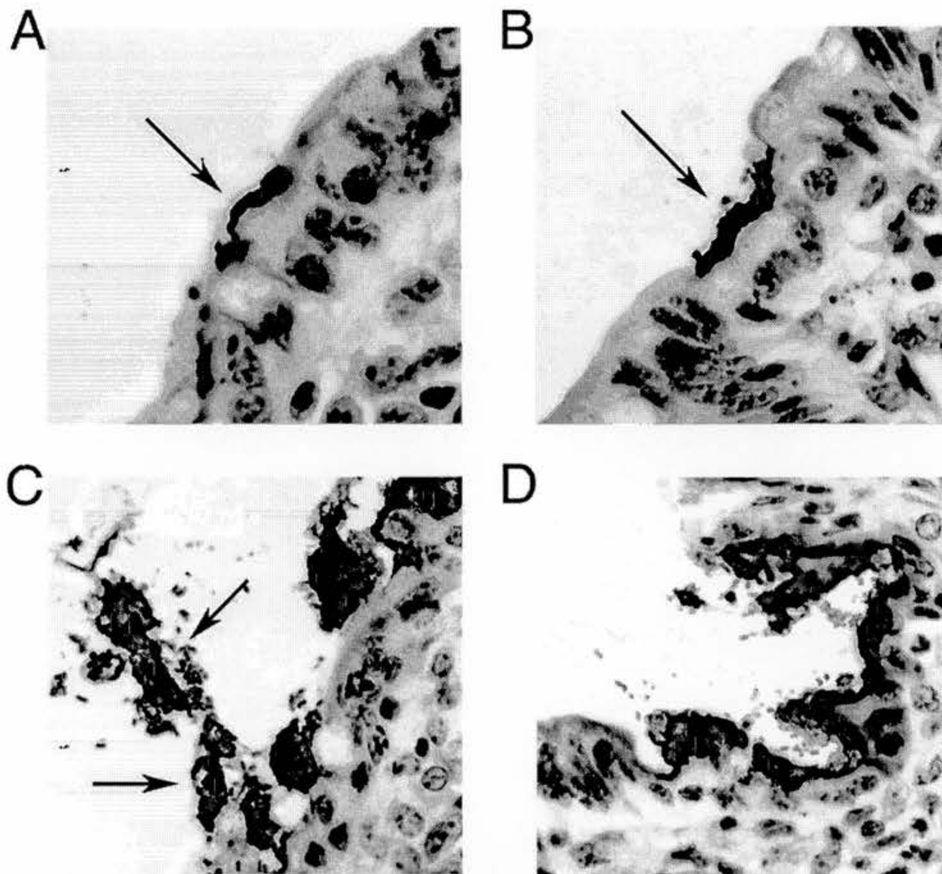

FIG. 1. Histopathological changes induced by *E. coli* O157:H7 colonization at the terminal rectum of cattle. (A) Apical surface of the enterocyte partly eroded by an *E. coli* O157 microcolony (arrow). (B) A larger *E. coli* O157 microcolony causing further erosion of the cytoplasm. An *E. coli* O157 colony is illustrated (arrow). (C) Enterocytes (arrows) heavily colonized with *E. coli* O157 are shown sloughing off from the basal membrane. Bacterial aggregates surround the enterocyte. (D) A larger *E. coli* O157 microcolony is shown on the lamina propria, following the shedding of the epithelial layer. Histopathology was carried out as described in Materials and Methods.

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application and 12 by oral administration. *E. coli* O157:H7 cell counts from tissue washings of the terminal rectum were significantly higher than from tissues of the large intestine ($P < 0.001$), irrespective of the challenge route. Significantly higher counts ($P < 0.01$) were detected in the other rectal sites (5, 10, and 20 cm proximal to the RAJ) compared to counts from tissue washings of large-intestinal tissues. For the animals challenged by the direct rectal administration method, *E. coli* O157:H7 bacteria were not recovered from bile or samples of digesta from nonrectal sites that included the rumen and the small or large intestine. For the orally challenged group, *E. coli* O157:H7 bacteria were not recovered from bile (data not shown). Terminal rectal tissue collected from colonized animals allowed us to study pathological changes.

Pathological changes at the terminal rectum. When bacterial concentrations exceeded 10^5 CFU per cm^2, *E. coli* O157:H7 bacteria could be readily detected in association with the epithelium by immunostaining and microscopy. In these positive tissues, at 15 to 21 days after challenge, the immunopositive bacteria were usually but not exclusively colonizing focal areas of the absorptive epithelium or the scarcer follicle-associated epithelium. Bacterial microcolonies ranged from those containing less than 30 bacteria to those with several hundred. The distribution of the colonies appeared random, with some microcolonies close together and others separated by large areas of noncolonized rectal tissue. In all cases, affected epithelial cells had effaced microvilli, and bacteria were intimately associated with their apical membranes. Occasionally, immunostained bacteria were present without producing major morphological alterations of the rectal epithelium. Generally, the mucosal border in foci with attached bacteria was low columnar to cuboidal (Fig. 1A and B). There was frequent exfoliation of the mucosal epithelium from the basal membrane, and bacteria were often seen in cavities of evacuated enterocytes (Fig. 1C and D). Groups of loose bacteria were also present in the mucus 40 to 100 μm from the intestinal surface and were not always associated with adherent microcolonies. On rare occasions, *E. coli* O157:H7 bacteria were also attached to areas of the squamous epithelium of the perianal region and crypts of the rectal mucosa. Rarely, an immunopositive bacterium was detectable along lymphatic lacteals of the lamina propria in areas lacking an epithelial surface. *E. coli* O157:H7 microcolonies were also detected by immunostaining at day 6, from rectal biopsy samples from three animals that were shedding $>10^5$ CFU g^{-1} of feces of *E. coli* O157:H7 bacteria. The distribution and intimate bacterial attachment to

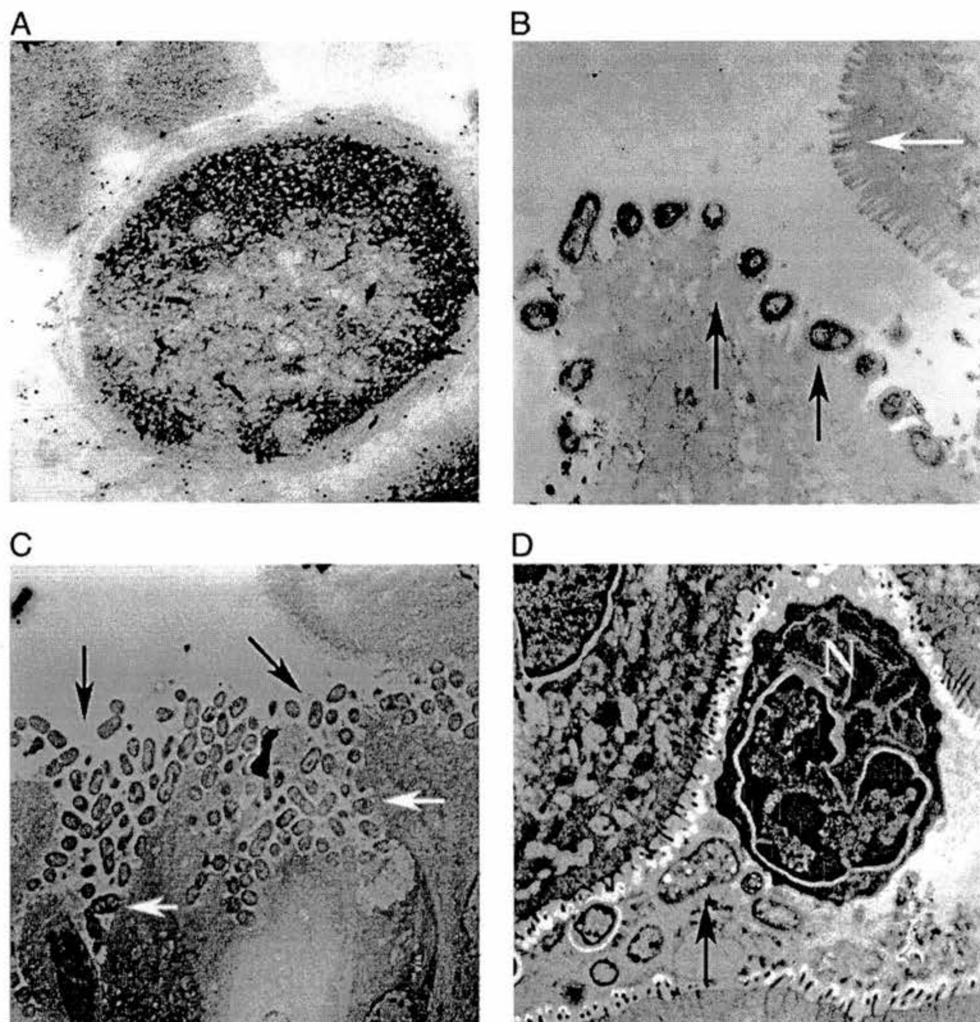

FIG. 2. Ultrastructure analysis of *E. coli* O157 interaction with the bovine terminal rectum. (A) Immunogold staining of *E. coli* O157 organisms. The image shows a single bacterium intimately attached through a pedestal to the host enterocyte, as part of an A/E lesion. Immunolabeling and TEM were carried out as described in Materials and Methods. (B) Cross-section of an *E. coli* O157 microcolony at the bovine terminal rectum. The bacteria are all intimately attached to the damaged epithelium (black arrows), inducing effacement of the microvilli. Unaffected brush border is visible on the neighboring cell (white arrow). (C) Aggregates of bacteria eroding the apical surface of colonized enterocytes (black arrows). Bacteria are intimately attached to the apical surface of the enterocyte (white arrows). (D) Extravasated polymorphic mononuclear leukocyte (N) adjacent to a bacterial cluster (black arrow).

enterocytes were similar to those observed for the cases examined at postmortem at days 15 and 21 postchallenge.

The pathological changes were further examined by scanning electron microscopy, which revealed multifocal clusters of rod-shaped bacteria of up to 2 μm in length, distributed randomly over the surface of the absorptive epithelium of the rectum. TEM studies and gold particle immunolabeling allowed us to identify *E. coli* O157:H7 bacteria on pedestals as part of A/E lesions (Fig. 2A and B). Pedestal heights varied but in some cases were up to 10 μm long. Some microcolonies appeared to consist of bacteria in layers, forming a stack, and individual bacteria were observed in the process of dividing while attached to the host cells. Bacterial microcolonies were associated with different degrees of enterocyte erosion (Fig. 2C). On occasion, granulocytes were present, interspersed among enterocytes, and exuded leukocytes formed aggregations in the gut lumen (microabscesses) (Fig. 2D). Lateral and basal membrane detachment and enterocyte exfoliation were often evident but present only when the bacteria had caused enough damage to approach the cell basal nucleus.

Cellular infiltration and inflammation. When *E. coli* O157:H7 was isolated from mucosal washings of tissues at levels higher than 10^5 CFU cm^{-2} or from biopsy samples taken from colonized animals that had similar numbers of *E. coli* O157:H7 bacteria in feces, there was a diffuse, low to mild granulocytic focal infiltration of the lamina propria of the rectum (Fig. 3A). In the terminal rectum, a significant ($P < 0.001$) leukocytic infiltrate was present in *E. coli* O157:H7-colonized animals (mean, 6.6 ± 1.4 per 0.25 mm^2) compared with controls (mean, 1.9 ± 0.9 per 0.25 mm^2). However, no differences were detected between the numbers of eosinophils, mast cells, and γδ T cells ($P > 0.65$, $P > 0.69$, and $P > 0.68$, respectively) in

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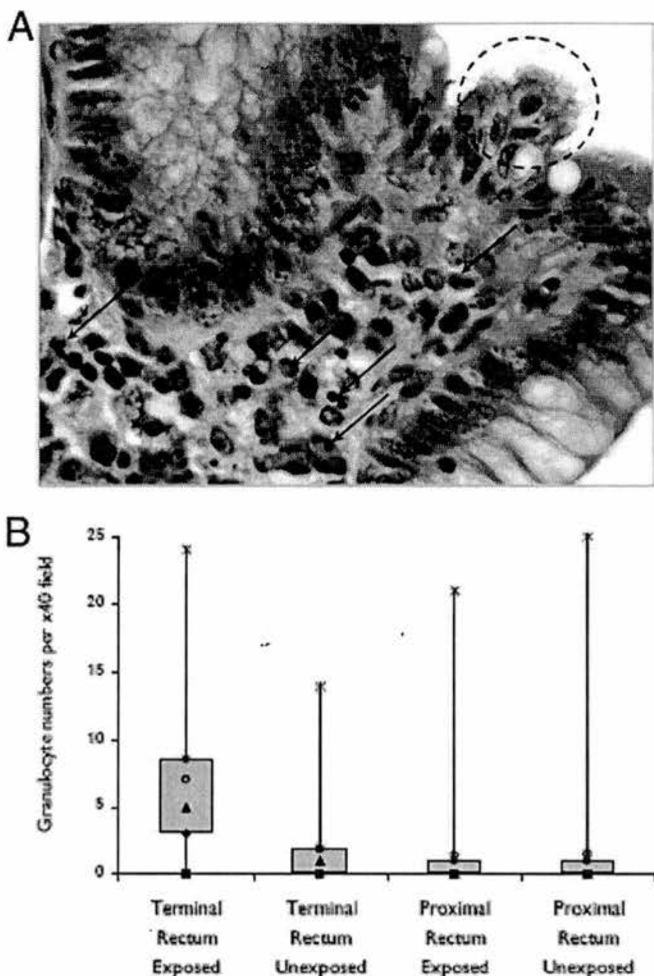

FIG. 3. Histological granulocyte quantification. (A) Hematoxylin-and-eosin-stained bovine rectal mucosa colonized with *E. coli* O157:H7. The dashed outline highlights colonized epithelium. Arrows indicate infiltration of granulocytes. (B) Box plots show the granulocyte counts in the terminal rectums of exposed and unexposed animals. The boxes contain 50% of the data, and the median count is illustrated by the black triangle. ◆, 1st quartile; ■, minimum; ▲, median; ○, mean; ×, maximum; ●, 3rd quartile. Samples were prepared and granulocytes identified as described in Materials and Methods.

colonized animals compared with those in control animals. For colonized animals, significantly more granulocytes ($P < 0.001$) were found in the terminal rectum than in the proximal rectum (+20 cm). However, in the area 20 cm proximal from the terminal rectum, no significant differences ($P > 0.42$) were detected between the neutrophil count of infected cases and that of noninfected cases (controls/mean of 1.5 ± 1.1 per 0.25 mm^2; cases/mean of 1.7 ± 1.0 per 0.25 mm^2) (Fig. 3).

Adaptive responses to *E. coli* O157:H7 colonization in the rectal mucosa. Mucosal antibodies were extracted from rectal mucosal homogenates to determine whether animals colonized by *E. coli* O157:H7 at the terminal rectum were generating specific mucosal antibody responses to the *E. coli* O157:H7 antigen. Samples were taken from three animals that shed *E. coli* O157:H7 at levels consistently higher than 10^4 CFU gram^{-1} for at least 2 weeks. In these samples, IgA antibodies were detected that bound to antigens of whole *E. coli* O157:H7 cells. Between 4 and 11 protein bands, with molecular masses ranging from 38 to 98 kDa, were recognized. The same homogenate samples showed no immune response to whole-cell extracts of the *E. coli* O26 or *E. coli* K-12 strains. Trypsinization of the *E. coli* K-12 and O157:H7 samples removed most of the immunoreactive material and resulted in the detection of a 14-kDa band for both strains (Fig. 4). No specific reactive bands were observed for four noninfected controls or from Western blots where the rectal mucosal homogenate was omitted (data not shown).

DISCUSSION

Work carried out by our group has demonstrated that *E. coli* O157:H7 has a tropism for the terminal rectum of cattle (25). The present study has confirmed this finding by examination of over 50 animals colonized by different challenge routes. The postmortem examination of these colonized animals also allowed the identification of minor sites of *E. coli* O157:H7 carriage. These sites included the rumen, small intestine, and most frequently, the proximal colon and, in particular, the lymphoid-rich tissue immediately distal to the ileocecal valve. In 2 animals out of the 54 studied, *E. coli* O157:H7 bacteria were distributed throughout the large intestine, given the even distribution of the bacteria throughout the fecal part. This finding is consistent with previous reports (25) and suggests that there is a different mechanism of colonization for a small number of animals, maybe due to the existence of multiple *E. coli* O157:H7 genetic types with different colonization strategies within one animal (11).

The postmortem and rectal biopsy materials collected from the colonized animals enabled a detailed study of the histological and ultrastructural changes associated with rectal colonization. A/E lesions were detected in animals with bacterial counts of more than 10^5 CFU g^{-1} in rectal tissues several weeks after experimental inoculation, and this is consistent with the previous finding that bacterial type III secretion system and A/E lesion formation are essential for the colonization and persistence of the organism in cattle (26). The long-term persistence is of a duration similar to the natural carriage observed for animals in field studies (1). The "shotgun" distribution of the microcolonies on the rectal mucosa may be caused by the dispersion of cells from the microcolonies into the surrounding environment, in the same manner proposed for *E. coli* spreading from biofilms (36) formed in response to shear forces and turbulent flow (8). In addition to A/E lesions, the major histopathological changes consisted of a reduction in enterocyte cellular width, a degeneration of cytoplasm in heavily colonized cells, and a frequent sloughing of enterocytes. These alterations were associated with a quantifiable neutrophilic response. The microscopic examination was made in animals shedding bacterial numbers similar to those animals considered super-shedders in field studies (10, 22, 34). Similar lesions have been reported in weaned calves 4 days postchallenge (7). Given the severe nature of the enterocyte changes observed, it is possible that most of the mucosal damage observed is due to enterocyte desquamation. In vitro studies have consistently reported decreased transepithelial resistance and opening of the tight junctions following *E. coli* O157:H7 colo-

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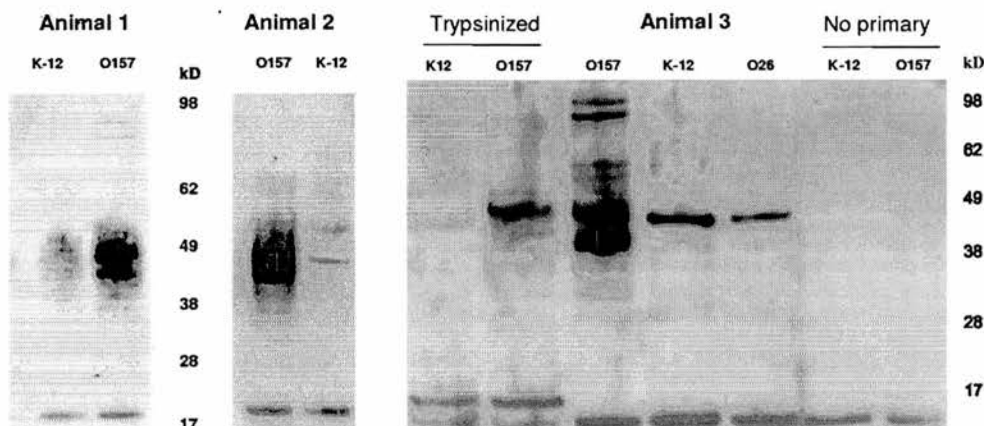

FIG. 4. Detection of mucosal antibody responses to protein antigens of *E. coli* O157. Mucosal antibody was obtained from homogenates of rectal mucosa sampled from three animals that had histopathological lesions detectable by microscopy. The homogenates were used to blot whole-cell preparations of *E. coli* strains O157 and K-12 and, in one case, O26. Multiple *E. coli* O157 immunoreactive bands were detected in comparison to either *E. coli* K-12 or *E. coli* O26. Trypsin digestion of the bacterial preparations removed most of the immunoreactive material. This is demonstrated with a blot using the homogenate from animal 3 (lanes labeled "Trypsinized"). Lanes marked "no primary" are controls containing *E. coli* O157 and K-12 preparations incubated with all the reagents, except for the rectal homogenate. Mucosal homogenates and Western blots were prepared as described in Materials and Methods.

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nization of monolayers (29), and similar findings have been reported in vivo in a mouse model of enteropathogenic *E. coli*/EHEC infection (13). The destructive alterations induced by *E. coli* O157:H7 infection in the bovine epithelial barrier induced a quantifiable neutrophilic response. This type of inflammatory reaction has been described previously in the intestinal tract for experimental infections of gnotobiotic or neonatal calves (0 to 1 day old) that are not immunocompetent. A quantified increase in neutrophils and eosinophils in the lamina propria has been described on one occasion (37), while other studies report similar increases in leukocytes in the colon and cecum (6) and sections of ligated ileal loops (32). In weaned calves, an eosinophilic infiltrate was reported for the perianal skin that bounds the rectal epithelium and in areas where A/E lesions were present (30). However, the quantified response in this study is the first for the predominant colonization site in experimentally challenged cattle.

The work also determined that *E. coli* O157:H7 infection induces a mucosal humoral immune response in cattle. Western blot analysis indicated 4 to 11 IgA-reactive bands of between 38 and 98 kDa. This pattern of bands was almost entirely removed by protein lysis of the bacterial cell sample. These responses may be the result of the high-level experimental challenge with *E. coli* O157:H7. However, this seems unlikely given that other experimental work has shown no immunological response to nonpathogenic strains of *E. coli* (14) and that challenge by *E. coli* O157:H7 leads to serological responses to proteins encoded by the locus of enterocyte effacement (3). This study has, therefore, provided evidence that cattle develop specific mucosal antibodies following colonization. Further studies and the application of more sensitive mucosal antibody detection methods (23) are required to determine whether these mucosal antibodies are involved in a protective immune response.

The terminal rectum is an area rich in lymphoid follicles (21), and it has been suggested that adherence to these sites may explain the tropism of *E. coli* O157:H7 bacteria for the bovine terminal rectum (25). In this study, extensive histological examination of terminal rectal tissues did not reveal a prominent association between *E. coli* O157:H7 microcolonies and follicle-associated epithelium. Thus, the reason for the terminal rectum tropism of *E. coli* O157:H7 is still obscure (19). Two of the main features of this area are a potentially reduced width of the mucous barrier, based on measurements taken with mice over Peyer's patches (33) and the fact that the recto-anal junction is adjacent to the anal sphincter. The combined effect of a reduced protective mucous barrier coupled with raised intrarectal pressure during defecation may facilitate colonization by the promotion of cell-to-cell contact that is one of the key mechanisms considered to induce type III secretion (31).

For many years, *E. coli* O157:H7 has been regarded as causing no clinical signs of infection in cattle. This study identifies pathological change and production of a local immune response in the terminal rectum in animals shedding high numbers of *E. coli* O157:H7 bacteria and infiltration of granulocytes and production of local IgA antibodies. This is the first report of local innate immune responses to *E. coli* O157:H7 rectal colonization in weaned calves, and so *E. coli* O157:H7 should not be regarded as a commensal organism in this host species. The findings may be of value in the development of methods for the control of *E. coli* O157:H7 carriage by cattle.

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