The immune response in cattle to virulence factors of enterohaemorrhagic *Escherichia coli*

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Declaration

The author performed the investigations and procedures described in this thesis unless indicated otherwise.

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Abstract

E. coli O157:H7 causes serious human disease including haemolytic uraemic syndrome and haemorrhagic colitis. Cattle are a known reservoir for *E. coli* O157:H7, but despite often-large levels of carriage, appear unaffected by the organism. Two important factors in the ability of *E. coli* O157 to cause disease are i) the possession of a 35Kbp pathogenicity island known as the locus of enterocyte effacement (LEE) and ii) the production of verotoxin. The products of the LEE interact bringing about intimate attachment to gut eneterocytes via the formation of attaching and effacing (AE) lesions.

The role of the bovine intestinal microbiota and immune system in protection against *E. coli* O157 associated disease is unclear. PCR and monoclonal antibodies were used to compare the distribution of the five *E.* coli LPS core types (R1-R4 + K12) within two populations of faecal isolates from healthy humans and cattle. To study the immune response in cattle LPS antigens were prepared and recombinant Intimin280 and EspB were cloned into the pGEX-4t_2 expression vector. The GST tagged proteins were purified by affinity chromatography. ELISA and immunoblot were used to examine the sera of healthy and experimentally infected cattle for antibodies reactive to antigens from *E. coli* O157:H7.

E. coli possessing R1 core LPS were most frequently detected in both human and cattle populations (50% and 40% respectively). Compared to the human

isolates a significantly higher level of bacteria with R3 core LPS was detected among the bovine commensal *E. coli* ($p<0.05$). In each population the highest level of antibody detected was reactive to the R4 core. In cattle the level of anti-R3 core antibody was significantly higher than the level of anti-R1, R2 and K12 antibody ($p<0.01$). This was not observed in humans.

Healthy adult cattle were found to have little or no antibody reactive to Int280. Prior to experimental challenge with *E. coli* O157, a number of calves had little or no detectable antibody to either Int280 or EspB; all other calves had detectable levels. The period of experimental infection did not alter the level of response in these animals. LPS was the only antigen found to elicit a response in any of the calves. The ability of the *E. coli* O157 inoculum to colonise the calves appeared unaffected by the levels of anti-Int280, EspB and LPS antibody detected. The antibodies present in the sera of the experimentally infected calves did not prevent the formation of AE lesions on HeLa cell monolayers

Antibody detected in calves prior to experimental infection may have been acquired from colostrum. However the lack of systemic responses to Int280 observed in healthy adult cattle would suggest that the response detected in calves originates from exposure to these antigens at birth. It is possible that the maturation of the commensal flora and local mucosal immunity in the calf reduces the level of systemic exposure to antigens such as Int280 and EspB and as a result the systemic response diminishes. Despite the presence of

antibodies reactive to Int280, EspB and LPS, *E. coli* O157 is still able to colonise calves yet disease is not recognised. The role of the bovine immune system in protection against *E. coli* O157 disease remains unclear.

Abbreviations

AE	Attaching effacing
cfu	Colony forming units
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothritol
eae	*E. coli* attaching effacing
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetracetic acid
EF-1	Elongation factor 1
EF-2	Elongation factor 2
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
Esp	*E. coli* secreted protein
ETEC	Enterotoxigenic *E. coli*
FCS	Foetal calf serum
Gb_3	Globotriaosylceramide 3
GST	Glutathione S-transferase
HC	Haemorrhagic colitis
HRP	Horseradish peroxidase
HUS	Haemorrhagic uraemic syndrome
Ig	Immunoglobulin alpha

IL	Interleukin
Int280	Intimin 280 (carboxy terminal 280 amino acids)
IPTG	Isopropyl β-D-thiogalactoside
KDa	Kilo Dalton
LB	Luria Bertani
LBamp	Luria Bertani suplemented with ampicillin
LBbroth	Luria Bertani broth
LEE	Locus of enterocyte effacement
LEEc	Locus of enterocyte effacement (*Citrobacter*)
LER	LEE encoded regulator
LPS	Lipopolysaccharide
LT	Heat-labile toxin
MRI	Moredun Research Institute
NC	Needle complex
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCP	Phenol chloroform petroleum
PCR	Polymerase chain reaction
PER	Plasmid encoded regulator
pfH_2O	Pyrogen free water
PMSF	Phenylmethylsulphonyl fluoride
pO157	Virulence plasmid of *E. coli* O157
RID	Radial immunodiffusion
SDS	Sodium doedecyl sulphate

SLT	Shiga-like toxin
ST	heat stable toxin
TAE	Tris-Acetic acid
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Tir	Translocated intimin receptor
TTBS	Tween-Tris buffered saline
UV	Ultra-violet
VT	Verotoxin
Vt2B	Verotoxin 2 B subunit
VTEC	Verotoxigenic *E. coli*

Introduction

1.1 *Escherichia coli* a brief history

Escherichia coli is a Gram-negative facultative anaerobic organism, forming large circular smooth colourless colonies on nutrient agar. Escherich first isolated 'Bacterium coli' in 1885 from the stools of infants, however the organism was subsequently re-classified under the genus Escherichia in 1920 (Parker & Duerden, 1990). Kauffman (1947) proposed the technique of typing *E. coli* upon the basis of the LPS (O-antigen), the capsule type (K-antigen) and flagella type (H-antigen). *E. coli* isolates are now routinely referred to on the basis of their O,H and K types. Today there are over 170 *E. coli* serotypes recognised and the genomes of *E. coli* K12 MG1655, *E. coli* O157:H7 EDL933 and *E. coli* O157 Sakai have all been fully sequenced (Blattner *et al,* 1997, Hayashi *et al* 2001,Ohnishi *et al*, 2001 and Perna *et al*, 2001).

1.2 Virotypes

E. coli implicated in diarrhoeal disease are often categorised into groups known as virotypes (virulence and types). Each group is made up of particular *E. coli* serotypes that share a common range of virulence determinants (Levine, 1987, Joseph *et al,* 2002). Virotypes include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC).

1.2.1 Enterotoxigenic *E. coli* (ETEC)

The ETEC are characterised by their ability to produce heat-labile (LT) exotoxin and two heat stable-toxins (STa & b) causing non-inflammatory diarrhoea (Joseph *et al*, 2002). The LT exotoxin is structurally similar and acts in a similar way to the main toxin of *Vibrio cholerae* causing massive fluid loss into the intestinal lumen (Guerrant *et al*, 1999).

1.2.2 Enteroinvasive *E. coli* (EIEC)

Disease caused by the EIEC shows similarities with shigellosis produced by *Shigella* spp. Plasmid encoded cell invasion factors allow the *E. coli* to invade and spread to adjacent cells (Joseph *et al*, 2002 and Guerrant *et al*, 1999).

1.2.3 Enteroaggregative *E. coli* (EAEC)

EAEC are so called due to their aggregative adherence patterns on Hep-2 cells (Joseph *et al*, 2002). Adherence is mediated by the plasmid encoded aggregative adherence fimbriae I and II (AAFI, AAFII) (Guerrant *et al*, 1999). EAEC are not known to secrete toxin but are associated with chronic or persistent diarrhoea (Joseph *et al*, 2002). The diarrhoeic effects of the EAEC may result from the production of a factor found to up-regulate interleukin-8 (IL-8) production in Caco-2 cells. The subsequent attraction of neutrophils and macrophages into the intestinal lumen may disrupt the epithelial barrier.

1.2.4 Enteropathogenic *E. coli* (EPEC)

EPEC have the ability to bind intimately to the surface of host cells and, via a type III secretion system, pass virulence determinants directly into the host

cell (Vallance and Finlay, 2000) The subsequent interaction of these bacterial proteins with the host cell lead to the formation of attaching and effacing lesions. These lesions are characterised by the effacement of microvilli and accumulation of polymerised actin beneath the site of bacterial adherence. This massive cytoskeletal rearrangement leads to the formation of a pedestal like structure (Vallance and Finlay, 2000). All of the components required to bring about AE lesion formation have been mapped to a 35Kbp pathogenicity island termed the locus of enterocyte effacement (LEE). The diarrhoea caused by EPEC is acute and non-bloody (Guerrant et al, 1999).

1.2.5 Enterohaemorrhagic *E. coli* (EHEC)

EHEC possess the LEE and share the ability to cause AE lesions on gut enterocytes. Since 1982 the EHEC have been implicated in a broad range of human diseases as a result of foodstuffs contaminated with faeces from animals colonised with EHEC and direct contact with infected animals (Nataro and Kaper 1998; Poxton, 1999; Chapman *et al*, 2000). Conditions arising as a result of EHEC infection include mild non-bloody diarrhoea, haemorrhagic colitis (HC), haemorrhagic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (Louie *et al*, 1999). As well as possessing the same virulence determinants as the EPEC, the EHEC have acquired the ability to produce verotoxin (**section 1.3**), which in some cases increases the severity of disease.

1.3 *Escherichia coli* O157:H7

1.3.1 The emergence of a new pathogen

Since its isolation from a woman with bloody diarrhoea in 1975 (Riley *et al*, 1983) *E coli* O157:H7 has become a major human pathogen. In 1982 two outbreaks of severe abdominal cramps with initially watery, progressing to grossly bloody diarrhoea affected at least 47 people. All were connected with having eaten similar meals at the same fast-food chains in Oregon and Michigan (Riley *et al*, 1983). Reports of similar sporadic outbreaks in both the USA and Japan had failed to identify the aetiological agent. Stool samples were examined for a variety of pathogens including *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium difficile* and *E. coli* (Wells *et al* 1983). A rare serotype of *E. coli*, O157:H7, was identified as the cause of the Michigan haemorrhagic colitis outbreaks. Although no *E. coli* O157:H7 was recovered from a cooked hamburger taken from one of the restaurants implicated in the outbreak, it was isolated from a frozen hamburger from the same outlet. The mechanism by which this organism caused disease was unclear. Since 1982 EHEC have been implicated in a number of large disease outbreaks. Over the past 11 years the number of reported cases of *E. coli* O157 associated disease in England and Wales have risen sharply from just over 200 reported cases in 1992 to over 1000 in 2001 (**Figure 1.1**). There is a definite seasonal variation in the number of cases reported with figures being higher in the spring and summer months than in the winter (**Figure 1.2**). The seasonal variation seen in human disease outbreaks is mirrored by the prevalence of *E. coli* O157 in cattle during the same months

(Hancock *et al*, 1994). This is possibly due to an increase in outdoor activities, children's camps and barbeques during the spring and summer. Owing to the elevated levels of *E.* coli O157 carriage among cattle and consequently the environment at this time, the risk of infection from either source is greater. Poorly cooked food and or contaminated water can lead to *E. coli* O157 associated disease.

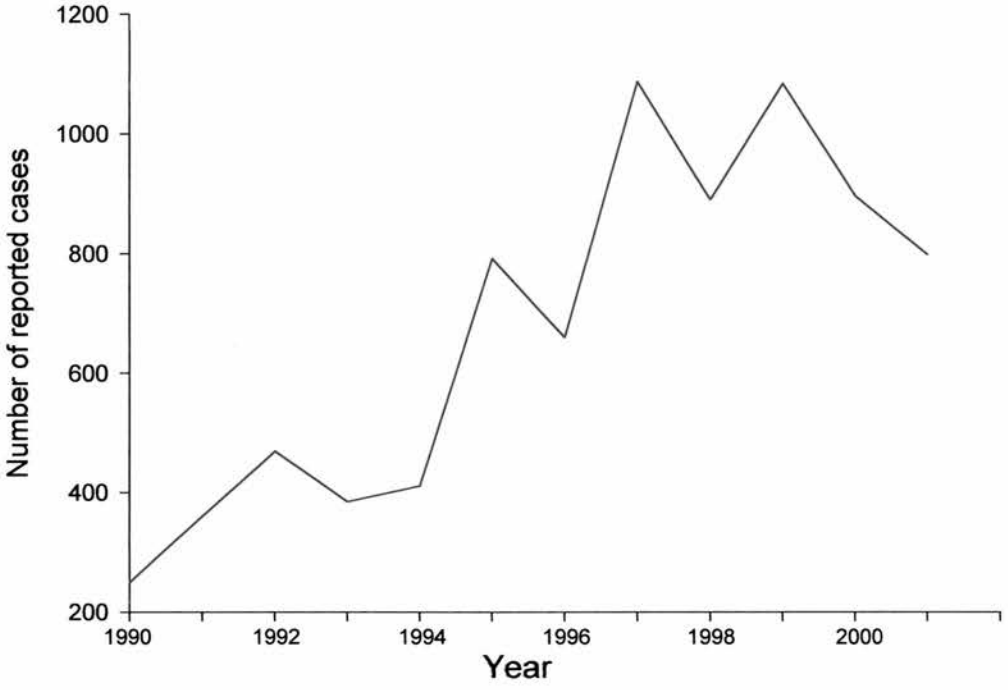


Figure 1.1: The number of reported cases of *E. coli* O157 infection over the last twelve years in England and Wales. Data from 2001 are preliminary. Information obtained from the Public Health Laboratory Service, UK (www.phls.co.uk)

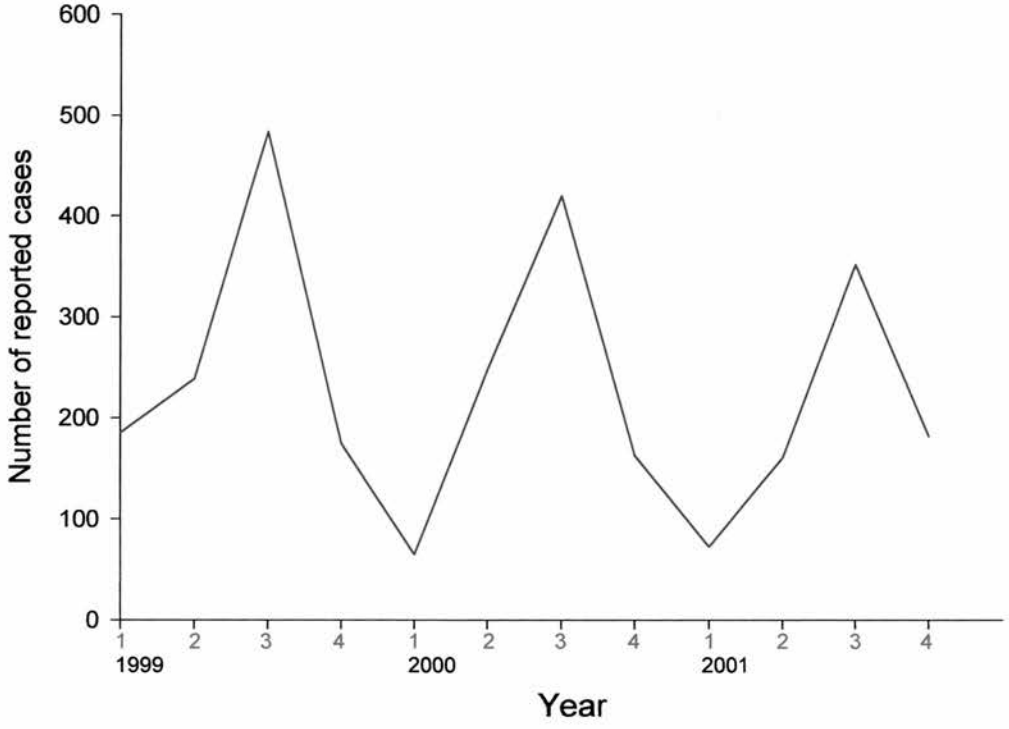


Figure 1.2: The number of reported cases of *E. coli* O157 infection over the last three years in England and Wales. Data show the number of cases reported each quarter. Data from 2001 are preliminary. Information obtained from the Public Health Laboratory Service, UK (www.phls.co.uk)

As detection techniques improve and people become more aware of the organism it is inevitable that the number of reported cases will continue to rise. Cases such as the UK's largest outbreak of *E. coli* O157:H7, which started on November 22nd 1996, attract massive media attention. A butcher's outlet in Lanarkshire, Scotland was identified as the source of the outbreak. Two hundred and seventy nine people were infected with the organism and 22 died as a result of infection (Cowden *et al*, 2001).

There is considerable debate as to whether or not *E. coli* O157 is an emerging pathogen, or whether in the past it was simply undiagnosed. Since the recognition of *E. coli* O157 as the causative agent of HUS and HC, collections of *E. coli* isolates associated with human disease prior to 1982 have been examined for the presence of *E. coli* O157. The Public Health Laboratory, UK reported only one *E. coli* O157 isolate among 15,000 strains typed between 1978 and 1982 (Day *et al*, 1983). Similar findings were reported in Canada (Johnson *et al*, 1983, Griffin and Tauxe, 1991). It is clear therefore that the rise in *E. coli* O157 associated disease since 1982 is real, and due to the recent emergence of this pathogen. A study by Avery *et al* (2002) used pulsed field gel electrophoresis and ribotyping to examine 207 *E. coli* O157 isolates. The combined use of the two techniques was able to group the 207 isolates into 146 types. This emphasises the enormous diversity that exists within the *E. coli* O157 serotype.

Although possessing identical virulence determinants to those of the EPEC, disease caused by EHEC is usually much more severe. Konowalchuk *et al* (1977) examined a number of ETEC, EIEC, and non-toxigenic *E.* coli strains and noted the ability of a number of ETEC strains to produce a toxin that had a very different cytotoxicity profile from LT and ST. The toxin was found to have a cytotoxic effect on Vero cells (African green monkey kidney cell line) only and was named VT (toxic to Vero cells). The toxin has subsequently been shown to be almost indistinguishable from the shiga-toxin of *Shigella dysenteriae* and hence is sometimes referred to as shiga-like toxin (SLT). The genes for verotoxin production are phage encoded. These organisms were grouped as the verotoxigenic *Escherichia coli* (VTEC). Since then a large number of serotypes have been identified as producing VT although not all have been associated with human disease. The EHEC are a further subdivision of the VTEC. EHEC possess the LEE pathogenicity island and the ability to produce one or more shiga-like toxin(s) (Perna *et al*, 1998).

1.3.2 Genetic diversity

The determination of the genome sequences of *E. coli* K12, O157 Sakai and O157 EDL933 have allowed detailed comparisons of the genomes of different *E. coli* (Ohnishi *et al*, 2001). *E. coli* K12 MG1855, *E. coli* O157 Sakai and *E. coli* O157 EDL933 all share around 4.1Mbp of DNA (Ohnishi *et al*, 2001). There is however considerable difference between the K12 strain and the two O157 strains, which is attributable to the large amount of strain

specific sequence in both (1.5Mbp in O157 Sakai and 1.34Mbp in O157 EDL933).

1.3.3 Evolution

The evolution of the EHEC from a possible EPEC origin has been examined in great detail. Feng *et al* (1998) proposed a model for the evolution of *E. coli* O157:H7. Using multilocus enzyme electrophoresis, the relationship between a number of O157 strains and other *E. coli* was investigated. Among 46 representative isolates, 15 electrophoretic types were found and close relationships between a number of O157:H7 and EPEC like O55:H7 isolates were noted.

Perna *et al* (1998) looked at sequence similarities between the LEE pathogenicity island of *E. coli* O157:H7 EDL933 and an EPEC strain. It was evident that there were significant differences between the two LEE sequences. While the average level of nucleotide identity was 93.9% many of the key genes involved in the formation of AE lesions, for example *tir*, (encoding the translocated intimin receptor), shared as little as 66% homology. Other genes showed a high degree of conservation especially those involved in type III secretion. This is strong evidence for the evolution of the two strains from a common ancestor and lends weight to the possibility that the EHEC evolved from the EPEC via the acquisition of the verotoxin encoding phage.

Citrobacter rodentium causes murine colonic hyperplasia. In extreme cases the disease is fatal but more often is associated with mild diarrhoea, coat ruffling and retarded growth. *C. rodentium*, like the EPEC and EHEC, possesses the LEE pathogenicity island and is able to produce AE lesions in infected mice (Newman *et al*, 1999). In contrast to the LEE of both EPEC and EHEC the LEE of *C. rodentium* (LEEc) was poorly characterised until very recently. Deng *et al* (2001) sequenced the entire *C. rodentium* LEE providing an insight into where the pathogenicity island may have come from. While LEEc was virtually identical to the LEE from EHEC and EPEC, in addition to insertion sequence elements at various point within LEEc, a number of genes had unique orientation. They also found that the LEEc was flanked by sequences that showed homology with a number of known plasmids. This was suggestive of a number of things, either the LEE was plasmid encoded, or the LEE had once been a mobile element in a plasmid and had become integrated into the chromosome. The detection of a number of insertion sequences within LEEc may suggest passage through a number of organisms. Deng *et al* (2001) suggested that *C. rodentium*, EPEC and EHEC had obtained the LEE pathogenicity island independently and by horizontal transfer among AE pathogens.

1.4 Transmission routes

While infection after ingestion of contaminated foodstuffs is well documented (Morgan *et al* 1993, Cowden *et al*, 2001, Keene *et al*, 1997), the question of whether or not direct contact between humans and animals could result in

infection has only recently been answered. Recent studies have utilised molecular typing techniques along with more established methods of EHEC typing such as phage typing, to investigate the transmission of EHEC strains from cattle to humans.

Louie *et al* (1999) investigated a number of human and bovine EHEC strains associated with disease outbreaks from two farms. Phage typing, verotoxin typing, pulsed field gel electrophoresis (PFGE) and plasmid profiling were used to identify any relationships between the outbreak isolates and a number of bovine strains isolated from cattle on each of the farms. They were able to show that a number of human isolates were of the same PFGE type as those isolated from cattle belonging to the outbreak farms. Chapman *et al* (2000) reported an incidence of infection in two children aged three and four after a visit to an inner city open farm. *E. coli* O157:H7 was cultured from faecal samples and swabs from cattle, pigs, sheep and goats. In addition, environmental sampling at the farm revealed extensive *E. coli* O157:H7 contamination. Molecular typing techniques confirmed that the strains isolated from the two children were of the same type obtained from the farm samples. It is clear therefore that direct transmission from animals to humans does occur.

1.5 The locus of enterocyte effacement (LEE)

The locus of enterocyte effacement (LEE) is a 35Kbp region of the genome of pathogenic *E. coli* such as EPEC and EHEC. The G+C content of this region (38.4%) is significantly lower than the rest of the genome and thus the LEE is likely to have been exogenously acquired (Vallance & Finlay, 2000). This pathogenicity island is responsible for the production of proteins crucial to the formation of attaching and effacing (AE) lesions. The pathogenicity island comprises 41 open reading frames which have been divided into five operons, *LEE1, LEE2, LEE3*, *LEE4* and *tir* the products of which interact bringing about the intimate attachment of EPEC or EHEC to gut enterocytes (Sperandio *et al*, 2000). **Figure 1.3** Shows the main features of the LEE and their location within the pathogenicity island.

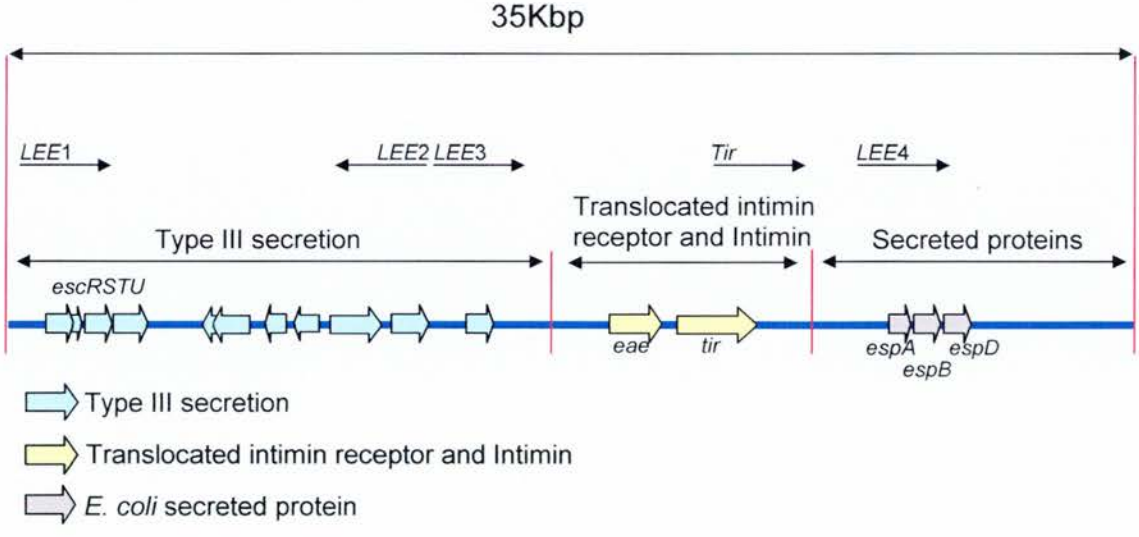


Figure 1.3: The locus of enterocyte effacement (LEE)

Adapted from Elliott *et al*, (2000)

1.5.1 LEE regulation

The genetic regulation of the LEE pathogenicity island is complex and poorly understood. There are a number of differences between the LEE of EPEC and of EHEC as discussed earlier. Briefly, while the LEE of EPEC and EHEC share 94% nucleotide sequence homology, a number of the individual genes can differ by up to 34% (Perna *et al* 1998). It is unclear however whether observed differences in EHEC and EPEC pathology are due solely to the sequence divergence of the LEE. EPEC and EHEC are known to differ at several other loci as well. McDaniel and Kaper (1997) showed that isolation of an EPEC O127:H6 derived LEE in a K12 background conferred the ability to produce AE lesions. It was concluded therefore that everything required to regulate and express the proteins involved in AE lesion formation must be contained within the LEE. Owing to the extensive sequence conservation between the LEE of EPEC and EHEC, it was assumed that the same would be true of the EHEC LEE. Elliott *et al* (1999) conducted the experiment and isolated an EHEC O157:H7 derived LEE in the same K12 background. They found however that the AE lesion phenotype was not conferred upon the recipient K12 strain. The inability of the EHEC LEE to confer the AE lesion phenotype on a K12 strain could have resulted from subtle differences in regulation between EPEC and EHEC LEE.

EPEC contain a large 70Kbp plasmid known as the EPEC adherence factor plasmid, this plasmid encodes a regulator of virulence genes known as the plasmid encoded regulator (Per) (Sperandio *et al* 1999). Per is known to

regulate the first gene in the *LEE*1 operon, the LEE encoded regulator (*Ler*) (Mellies *et al*, 1999). EHEC lack the EPEC adherence factor plasmid and therefore the LEE may be considered to be under different regulation. *Per* however is obviously not essential to the expression of the LEE genes as it was not required to confer the AE lesion phenotype in the K12 background.

As well as controlling the expression of the type III secretion system, secreted proteins, intimin and Tir, *Ler* is also thought to control the expression of a number of other genes not involved in AE lesion formation. Sperandio *et al* (1999) reported that quorum sensing also plays a role in the regulation of the LEE operons in EHEC and EPEC. Although EHEC and EPEC have a low infectious dose, quorum sensing molecules produced by other commensal *E. coli in vivo*, may activate the LEE pathogenicity island. Not only are there differences in regulation between the EPEC and the EHEC but regulatory differences have also been noted in different EHEC strains. McNally *et al* (2001) noted that the levels of secreted proteins expressed by different *E.* coli O157 strains were often very variable.

1.6 Type III secretion

The genes encoding the type III secretion system, responsible for translocating the AE lesion effector molecules into the host cell are located on the LEE pathogenicity island. Several genes termed *sepA, B, C and D* were shown to have homology to the genes involved in type III secretion systems of other organisms such as *Shigella* and *Yersinia* species (Jarvis *et*

al, 1995). Subsequently a number of other genes involved in type III secretion have been identified; these are the *esc* and *ces* genes (Elliott *et al*, 2000).

1.7 *E. coli* secreted protein A (EspA)

E. coli secreted protein A (EspA) forms long filamentous structures on the surface of EHEC and EPEC connecting the type III secretion system directly to the host cell, acting much like the needle of a syringe through which other virulence determinants pass. Knutton *et* al (1998) showed that EspA was able to form a large extracellular filamentous complex that is required to translocate proteins from the bacterium to the host cell. By culturing EPEC strains in Dulbecco's modified Eagle's medium (DMEM) large filamentous structures that reacted with anti-EspA antibody were observed. Homology between the coiled domains of bacterial flagellins, essential to the formation of flagella, and the carboxy-terminus of the EspA protein also pointed to its involvement in the formation of these large filamentous structures (Delahay *et al*, 1999). The exact nature of the interaction between EspA and the needle complex (NC) of the type III secretion system was unclear. By comparing the NC preparations of *Shigella* with those of several EPEC, Sekiya *et al* (2001) showed that it was evident that the EPEC NC was far longer than that observed in *Shigella*. This was due to the association of EspA proteins at the end of the needle complex, which resulted in the formation of vast extended needles of almost 600nm in length (**Figure 1.4**). By disrupting EscF, a protein crucial to the formation of the needle complex

in type III secretion, Sekiya *et al* (2001) were able to show that the formation of the EspA filaments was completely disrupted. Thus in order to form the long EspA filaments, association of EspA proteins with the type III secretion apparatus, in particular the needle complex, is crucial.

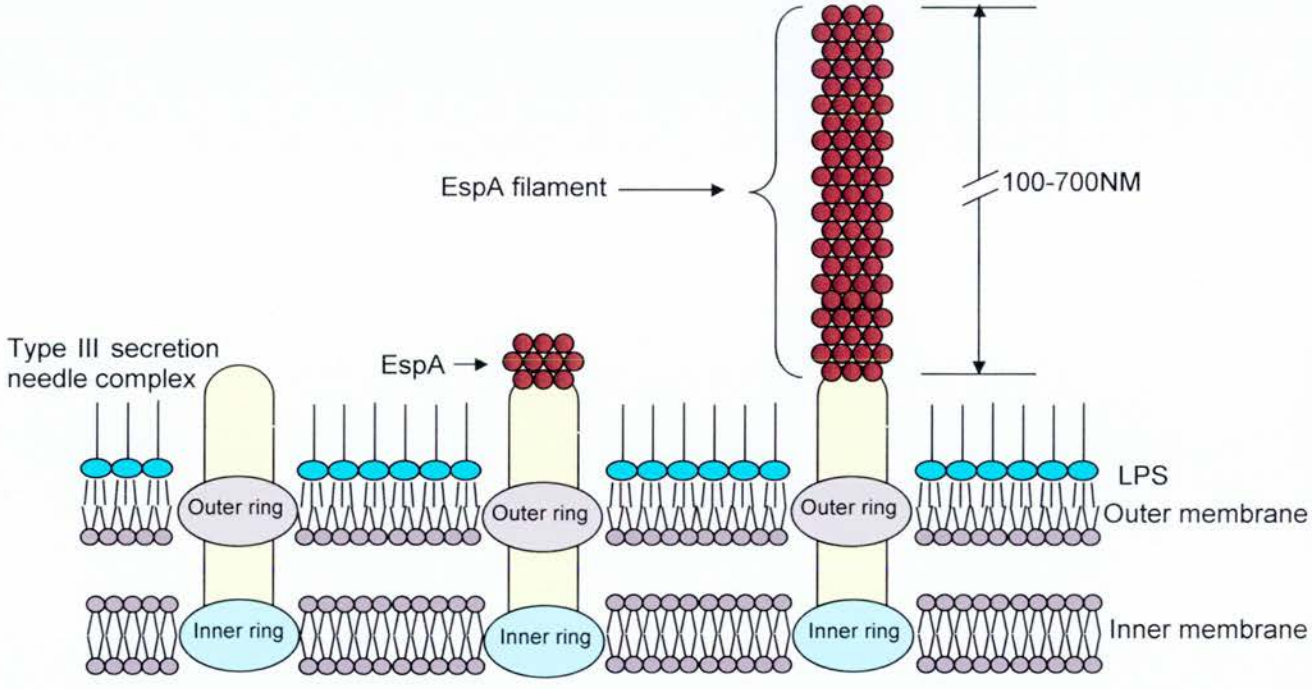


Figure 1.4: The formation of EspA filaments and their association with the type III secretion system. Adapted from Sekiya *et al* (2001)

Neves *et al* (1998) investigated a number of EPEC strains and found that the *espA* gene was heavily conserved between strains. One serotype was found to contribute most of the diversity observed and when compared along side the other EspA proteins was found to be less immunogenic and also when polymerised at the bacterial surface, a lot shorter.

1.8 The translocated intimin receptor (Tir) and intimin

Intimin is a 94-97KDa outer-membrane protein largely responsible for the attachment of EHEC (and other LEE containing organisms) to gut enterocytes (Kenny *et al*, 1997). When first discovered no function was assigned to intimin, but homology with the invasin of *Yersinia* implicated intimin in a receptor-binding role (Frankel *et al*, 2001). The homology between the two proteins was localised to the amino-terminus, this shifted attention to the carboxy - terminus which possibly contained receptor binding activity. Attention was focussed on a 280 amino acid region of the carboxy-terminus, which was shown to interact with HEp-2 cells (Kelly *et al*, 1999). This 280 amino acid fragment is widely known as intimin280 (Int280). As well as binding to EHEC/EPEC activated HEp-2 cells, an EHEC/EPEC independent binding of the truncated intimin fragment was also observed implicating a cell bound receptor with a degree of specificity for the intimin protein.

A 90KDa protein termed Hp90 was identified as the receptor for intimin. Hp90 was first assumed to be a host cell protein that had intimin binding activity but

was soon recognised as a protein of bacterial rather than mammalian origin (DeVinney *et al*, 1999). It was apparent that Hp90 like intimin, was a LEE encoded protein that was exported via the type III secretion system, through the EspA filament, to the host cell, where upon it became integrated into the host cell membrane to act as a receptor for Hp90 was renamed the translocated intimin receptor (Tir).

The structure of intimin280 (residues 658-939) has been determined as comprising of three globular domains (Kelly *et al*, 1999). Domain 2 (D2; residues 658-751), domain 3 (D3; residues 752-841) and domain 4 (D3; residues 842-939) make up the extracellular portion of intimin that binds Tir (**Figure 1.5:**Luo *et al*, 2000). More recently the binding domain was narrowed to the terminal 150 amino acids at the carboxy-terminus (Kelly *et al*, 1999), which encompasses the lectin like D4, which possesses the Tir binding domain (Luo *et al*, 2000). A 76 amino-acid loop in D4 has been identified as being essential for intimate attachment of EPEC/EHEC to cultured mammalian cells (Frankel *et al*, 1994)

Analysis of the Tir sequence revealed that the protein had two transmembrane domains. This implied that the protein took on a hairpin like structure with the central portion possessing the intimin binding activity (Frankel *et al*, 2001). **Figure 1.5** shows how intimin and Tir interact to bring about intimate attachment during AE lesion formation.

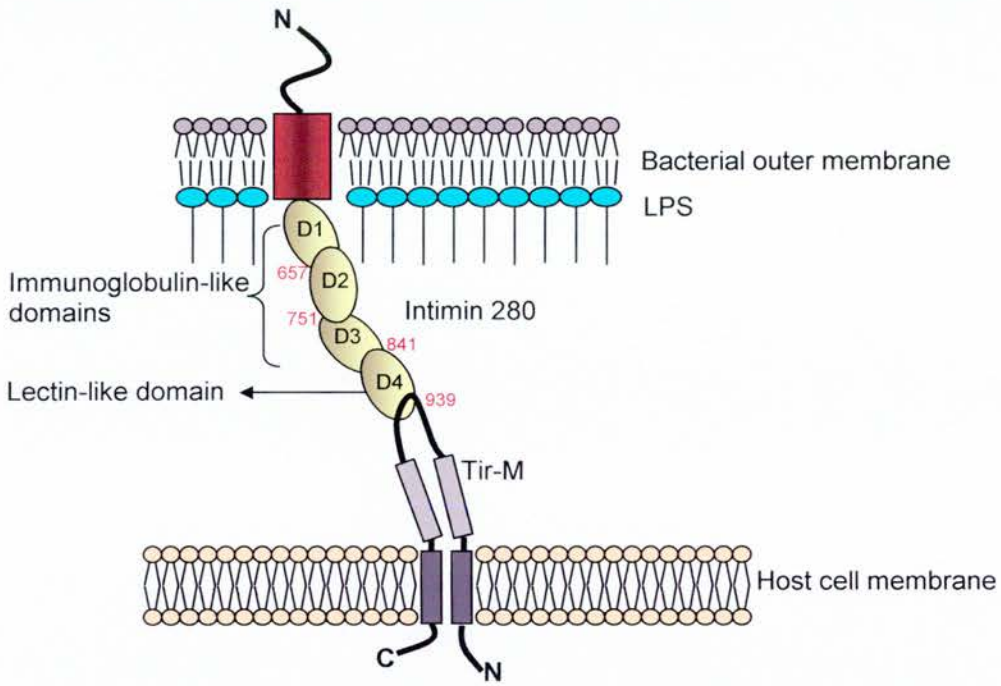


Figure 1.5: The interaction of Intimin and the translocated Intimin receptor Tir in the formation of AE lesions. Tir-M: Translocated intimin receptor middle section.

Adapted from Luo *et al* (2000)

1.9 Intimin types α β γ δ ε

While intimin maintains a high degree of homology within the N-terminal region, there is considerable variation within the carboxy-terminus leading to the recognition of intimin types (Frankel *et al,* 2001*)*. At least five distinct intimin types have so far been found. Intimin α, β, γ, δ and ε have all been found to associated with different EPEC and EHEC (Fitzhenry *et al*, 2002). EPEC have evolved into two groups of related clones, termed EPEC 1 and 2 (Adu-Bobie *et al*, 1998). Each group spans a range of O-antigen types but the flagella (H-antigen) types do not vary greatly. Intimin ε was recently identified as a new variant (Oswald *et al*, 2000).

Intimin α is associated with the group 1 EPECs (EPEC1) (Adu-Bobie *et al*, 1998). Intimin β was found in both EHEC and EPEC strains while intimin γ is expressed by *E. coli* O157:H7. This apparent preferential association with selected *E. coli* serotypes led to the suggestion that intimin type may influence the pattern of colonisation and tissue tropism exhibited by a particular LEE positive *E. coli* (Phillips *et al*, 2000). Phillips *et al* (2000) were able to show that an EPEC strain expressing intimin α formed AE lesions on *in vitro* organ culture proximal small intestine, distal small intestine and the follicular associated epithelium of the Peyer's patch. However an EHEC strain expressing intimin γ was found to bind and cause AE lesions on the follicular associated epithelium of the Peyer's patch. By deleting *eae* from an *E.* coli O157:H7 strain and complementing with either γ or α *eae*, Fitzhenry *et al* (2002) showed that the ability of *E. coli* O157:H7 to bind to various *in vitro*

organ culture tissue explants, was dependent upon the intimin type. Expression of intimin α allowed *E. coli* O157 to bind to both Peyer's patch and small intestine explants. Intimin γ expression however limited the binding ability of the same *E. coli* O157 to the Peyer's patch. Preferential binding of EHEC/EPEC to the Peyer's patch may be the result of the expression of microfold (M) cells. Their role as antigen sampling cells may act as a route into the host (Fitzhenry *et al*, 2002).

The essential role intimin plays in disease was demonstrated by Dean-Nystrom *et al* (1998). Colostrum deprived calves received either intimin positive or intimin negative strains of *E. coli* O157:H7. It was found that the presence of the *eae* gene was crucial to the organism's ability to cause disease.

1.10 The immunogenicity of intimin280

Adu-Bobie *et al* (1998) were able to elucidate which regions of the carboxy terminal 280 amino acids of intimins α and β were immunogenic. In the first instance a human colostral IgA and rabbit polyclonal antiserum were used to detect the immunodominant regions of the various Int280 fragments. Results showed that there were two immunodominant regions. The first lay within the first 80 amino acids and the second mapped to a 50 amino-acid segment between residues 80 and 130 of both intimin types. Further investigation identified the first 20 amino acids of Int280α and the terminal 20-80 residues of Int280β as being the specific regions responsible for antibody reactivity. It

is possible that conformational changes induced by protein tags used in this study affected the immunogenicity of a number of the protein fragments and resulted in the observed differences.

1.11 Actin polymerisation

Characteristic of AE lesion formation is the accumulation of polymerised actin beneath the site of EPEC/EHEC adherence (Celli *et al*, 2000). The cytoskeletal rearrangements contribute to the formation of the pedestal-like structure upon which the bacteria sit. Finlay *et al* (1992) investigated the recruitment of microfilament-associated proteins within these pedestal structures. Actin, α-actinin, talin and ezrin were all found localised beneath areas of bacterial adherence. Talin and ezrin are two microfilament associated proteins that have been implicated in linking actin filaments to transmembrane receptors thus the results suggest that the polymerised actin fibres may be associated with Tir (Finlay *et al* 1992).

Rosenshine *et* al (1996) showed that three proteins designated Hp39 (39KDa), Hp72 (72KDa) and Hp90 (90KDa: Tir) were all tyrosine phosphorylated following HeLa cell infection with EPEC. Further work by Rosenshine *et al* (1996) showed that this heavy accumulation of tyrosine-phosphorylated proteins occurred directly beneath the site of EPEC adherence. They postulated that this may be an important role in steps leading to the polymerisation of actin and subsequent pedestal formation. However Ismaili *et al* (1995) were able to demonstrate that in EHEC induced pedestals there was no accumulation of tyrosine phosphorylated proteins

beneath the surface of the adhered bacteria, yet the same cytoskeletal rearrangements took place. Furthermore DeVinney *et al* (1999) confirmed that the Tir of EHEC was not tyrosine-phosphorylated. These results show that tyrosine-phosphorylation is not a necessary precursor to the formation of EHEC induced pedestals in AE lesions.

1.12 *E. coli* secreted proteins

The LEE encoded secreted protein genes encode a number of proteins crucial to signalling events leading to AE lesion formation. These signals include activation of phospholipase C-γ, NF-κB induction and changes in membrane potential (Taylor *et al,* 1998). The secreted proteins may be detected in the culture supernate of certain *E. coli* strains (Wainwright *et al*, 1998). The secreted proteins are transported via the LEE encoded type III secretion system, through the EspA filament and into the host cell whereupon a number of events result in actin polymerisation and the formation of an AE lesion (McNally *et al*, 2001).

1.12.1 EspB and D

EspB is an integral protein involved in AE lesion formation. Without EspB no alterations in the host cell cytoskeleton or inositol phosphate flux are observed (Tacket *et al*, 2000). Taylor *et al* (1998) reported that EspB was targeted to the cytoplasm of HeLa cells in a mechanism that was clearly dependent upon EspA and EspD function. Intimin knockout strains had no affect on the ability of EspB to enter the HeLa cell and localise in the

cytoplasm. This was confirmed by Wolff *et al* (1998) who showed that HeLa cell contact via intimin and/or the bundle forming pilus was not required for complete translocation of EspB, although translocation was not as efficient as with the wild type strain. The mechanism by which EspB was able to be translocated from the bacteria and accumulate in the cytoplasm of HeLa cells was at the time unclear. Wainwright *et al* (1998) were able to show that in common with the type III secretion systems of other organisms, for example *Yersinia* and *Shigella*, a specific chaperone protein was required to effect EspB and D secretion. By mutational analysis it was determined that a protein termed CesD was absolutely required for the secretion of EspD and its absence dramatically affected the level of EspB secretion. CesD showed significant homology to a number of proteins already implicated in type III secretion, for example SicA (*salmonella* invasion chaperone) from *Salmonella typhimurium* and IpgC (Invasion plasmid gene) from *Shigella flexneri* (Wainwright *et al* 1998). Since EspB secretion was still observed it is likely that chaperone proteins are not solely responsible for the export of these proteins and that other factors are involved.

Work by Wolff *et al* (1998) showed that *espB* mutants were unable to secrete EspB even when complemented with a plasmid with the intact *espB* gene. This implied that actual expression of the *espB* gene was required to allow EspB translocation. The same study also expanded on work by Taylor *et al* (1998) and proved that EspB was not only localised to the HeLa cell cytosol, but could also be detected in the membrane fractions. Homology between

EspB and the *Yersinia* outer membrane protein B (yopB) involved in the formation of a translocation channel in the host cell membrane, suggests that EspB could have a similar role (Frankel *et al*, 1998).

Analysis of the *espD* sequence revealed homology with a number of proteins involved in type III secretion for example IpaB of *Shigella* and SipB of *Salmonella* (Wachter *et al* 1999). The structure of EspD is predicted to contain two coiled-coil domains as well as two transmembrane domains (**Figure 1.6**: Wachter *et al* 1999). Just as with the EspA protein, the coiled-coil domains suggest a role in protein-protein interactions. Daniell *et al* (2001) were able to show that these coiled-coil domains did indeed bring about strong EspD-EspD reaction

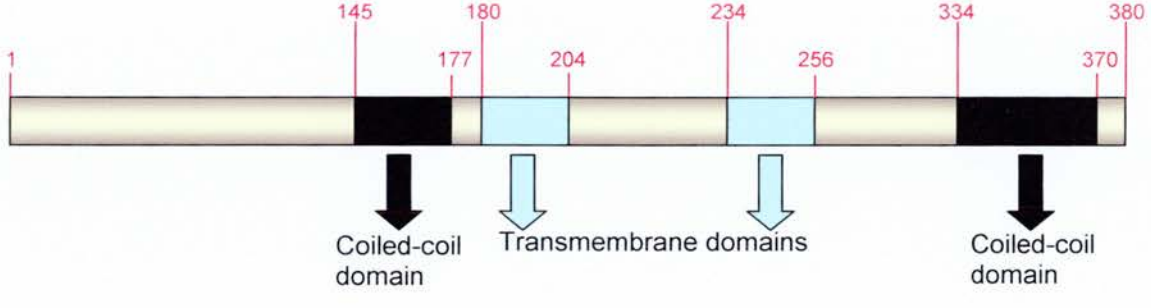


Figure 1.6: Structure of EspD showing the position of the coiled-coil domains (residues 145-177 and 324-370) and the transmembrane domains (residues 180-204 and 234-256) Adapted from Daniell *et al* (2001)

EPEC infection of HeLa cells by Wachter *et al* (1999) showed that like EspB, EspD could be found integrated into the host cell membrane. By preparing glutathione-S-transferase tagged EspB, Ide *et al* (2001) were able to show that EspD interacted with EspB presumably via the coiled-coil domains. With the use of electron and atomic force microscopy they were also able to demonstrate the formation of pores in sheep red blood cell membranes and revealed in exquisite detail the shape of the EspB/D pore structures. The pores appear to result from the interaction of 6-8 units (**Figure 1.7**). The opening of the pore was gauged at approximately 8nm, which would allow the EspA filament complex at around 2-3nm, to be inserted into the pore.

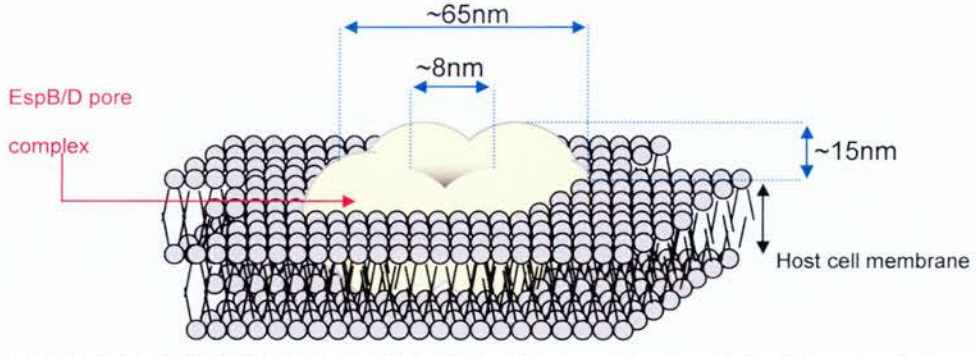


Figure 1.7: The EspB/D pore complex. Sizes shown were ascertained by atomic force microscopy.
Adapted from atomic force microscopy image, Ide *et al* (2001)

It is clear that the coiled-coil domains of EspA, B and D allow each of the proteins to interact. Firstly the formation of the long EspA filament allows effector proteins such as EspB and D to be translocated to the host cell were upon they associate with one another and form a pore into which the EspA filament engages. Intimin and/or bundle forming pilus mediated attachment were important for full translocation efficiency (Wolff *et al* 1998, Taylor *et al* 1998).

Wolff *et al* (1998) hypothesised that attachment via intimin stabilised the fragile EspA filaments and allowed effective translocation. However it is clear that in order for intimin to bind, Tir must already have passed through the EspA filament, into the host cell and have localised to the host cell surface, a process downstream of, and dependent upon, EspB translocation (Kenny *et al* 1997). *In vivo* there is therefore a requirement for EspB translocation without intimin/Tir interaction. Therefore the ability of intimin to bind β-1 integrins (Frankel *et al*, 1996) may be vitally important for the initial translocation of EspB/D prior to a stable intimin/Tir interaction.

1.12.2 *E. coli* secreted proteins F, G, C and P

There are a number of other proteins secreted by EHEC and EPEC about which less is known, these include *E. coli* secreted protein F (EspF) involved in the disruption of the tight junctions of gut enterocytes (Elliot *et al,* 2002), and *E. coli* secreted protein G (EspG) which shows homology to the VirA protein of *Shigella flexneri* (Elliot *et al*, 2001). Although the role of VirA in the

pathogenesis of *Shigella flexneri* is unclear it is known to be important in cell invasion and could play a similar role in *E. coli*.

E. coli secreted proteins C and P (EspC and EspP) are not associated with the LEE pathogenicity island and their secretion is not dependent upon the type III secretion system utilised by the LEE encoded secreted proteins (Mellies *et al* 2001 and Brunder *et al* 1997). The gene encoding EspC has been mapped to a region of the chromosome demonstrating the characteristics of a pathogenicity island (Mellies *et al*, 2001). This region of DNA was not found in either commensal or laboratory strains of *E. coli*. EspC is a 110KDa protein and earlier work by Stein *et al* (1996) had identified significant homology between EspC and a number of other proteins including a group of immunoglobulin A protease-like proteins. The IgA proteases of other organisms are known to mediate their own export from cells (Mellies *et al*, 2001). These autotransported proteins comprise a large group of proteins many of which are not IgA proteases, but do possess a serine protease motif (Mellies *et al*, 2001). Stein *et al* (1996) reported that although EspC showed IgA protease homology, it was unable to cleave human IgA. Also by mutating *espC*, Stein *et al* (1996) showed that EspC was not required for the formation of AE lesions by EPEC. The role of this protein is unclear.

EspP is a large protease (1300 amino-acids, 104 kDa) encoded by a 3.9Kbp region of the *E. coli* O157 virulence plasmid, pO157 (Brunder *et al*, 1997). This 90Kbp plasmid also encodes the EHEC-haemolysin (Bauer and Welch,

1996). The role of EspP in disease is unclear, but anti-EspP IgG responses detected in sera from patients infected with *E. coli* O157, suggests that it is expressed during infection (Brunder *et al* 1997).

1.13 Lipopolysaccharide

1.13.1 Structure

Lipopolysaccharide (LPS: endotoxin) is an integral part of the outer membrane of all Gram-negative bacteria (Poxton, 1995). Exposure to endotoxin can lead to potentially fatal systemic inflammatory reactions (Erridge *et al*, 2002). The whole LPS structure of *Escherichia coli* (and other related species) may be divided into three distinct regions (**Figure 1.8**), the hydrophobic Lipid A moiety, responsible for the endotoxic properties of LPS (Galanos *et al*, 1985) the core oligosaccharide and the O-polysaccharide antigen (Erridge *et al*, 2002).

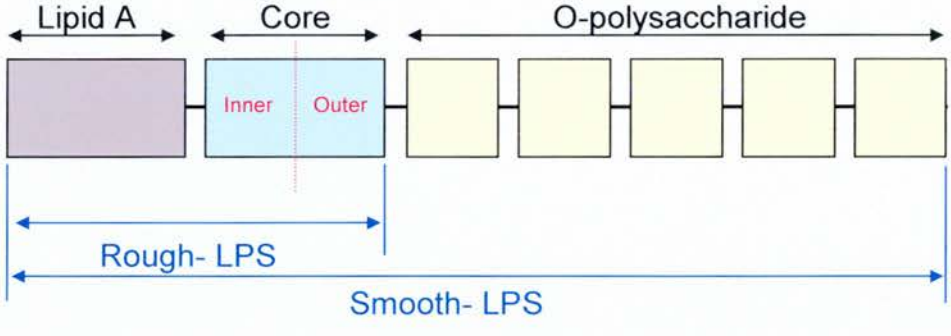


Figure 1.8 LPS schematic showing the three structural regions and the components of rough form and smooth form LPS

LPS may occur in one of two forms, rough (R-form) or smooth (S-form) (Poxton, 1995). S-form LPS comprises all three structural regions shown in **Figure 1.8**, while R-form LPS is characterised by the loss of the O-polysaccharide leaving just the Lipid A and the core region (Amor *et al*, 2000). Variability in the LPS structure ranges from the Lipid A region which is highly conserved to the O-polysaccharide of which there are over 170 types

known in the *E. coli* alone (Amor *et al*, 2000). There are five known *E. coli* core types denoted R1, R2, R3, R4 and K12. The core region may be divided into inner and outer core. The inner core comprises three 3-deoxy-D-*manno*-octulosonic acid (KDO) and three L-*glycero*-D-*manno* heptose (hep) residues and displays little variation between each of the five core types. The variation observed in the outer core region is responsible for the differences in the five core types. **Figure 1.9** shows in detail the structure of *E. coli* LPS. Complete core LPS comprises the lipid A, three KDO and three heptose residues along with five (six in the case of K12) variable sugars dependent upon core type (**Figure 1.9b**), this is known as Ra complete core LPS. There are several forms of incomplete core LPS denoted Rb, Rc, Rd and Re, each comprising progressively less of the complete core structure. The components of Rb, Rc and Re LPS are shown in **Figure 1.9a**. The high degree of conservation within the membrane proximal parts is possibly due to the essential role that core LPS plays in membrane stability (Heinrichs *et al*, 1998).

1.13.2 O-antigen

Exposed at the bacterial surface, the O-polysaccharide is easily accessible to the host immune system and leads to the generation of O-specific immune responses, thus the term O-antigen is commonly used (Erridge *et al* 2002). The O-antigens appear at the cell surface as chains of varying length (Poxton, 1995). This is demonstrated by the O-antigen 'ladder pattern' observed when smooth-form LPS is resolved by polyacrylamide gel electrophoresis. Each band in the ladder results from the addition of one

extra O-antigen unit to the core (Poxton, 1995). The enzymes required to assemble the O-antigen chains have been mapped to a region of the *E. coli* chromosome known as the *rfb* locus (Schnaitman and Klena, 1993).

While core LPS is crucial to membrane stability, the O-antigen plays a vital role in protection against the effects of a number of otherwise lethal compounds and substances. The O-antigen provides an effective barrier against hydrophobic antibiotics, phagocytosis, complement, free fatty acids and detergent (Burns and Hull, 1998). There are over 170 *E. coli* O-antigen serotypes recognised, yet only a few have been implicated in disease. These include O1, O2, O4, O6, O7, O8, O15, O18 and O75 most often associated with blood borne and urinary tract infection (Gibb *et* al, 1992, Burns and Hull, 1998). The ability of certain serotypes to cause disease is related to the degree of serum resistance the O-antigen confers (Porat *et al*, 1992). Complement mediated killing (serum sensitivity) requires that the membrane attack complex has access to the bacterial membranes (Erridge *et al*, 2002), various O-antigens appear to function well as barriers against this, while others do not.

Porat *et al* (1992) compared the lengths of O-antigen side chains of serum resistant *E. coli* and serum sensitive *E. coli*. By constructing liposome-radioactive-chromium packages incorporated with O-antigen LPS derived from serum sensitive and resistant strains, it was possible to determine the complement resistance offered by each O-antigen type. They observed a

correlation between the length of the carbohydrate side chains and the serum resistance of particular O-antigens. O-antigens found to be serum resistant had longer carbohydrate side chains than those that were serum sensitive. It is possible that serum sensitivity and resistance of *E. coli* is far more complex than simply a matter of which O-antigen is expressed and the length of carbohydrate side chains. Other studies have reported only minor alterations in serum sensitivity upon the loss of the O-antigen (Russo *et al*, 1995).

Being the dominant antigen at the bacterial cell surface, the O-antigen may be expected to play an important role in adherence. However the role (if any) of the O-antigen of *E. coli* O157:H7 in enterocyte binding is unclear. Studies by Bilge *et al*, (1996) showed that a mutant strain unable to express the O-antigen was able to bind to HeLa cells. When the ability of the mutant to synthesise the O157 antigen was restored, the binding affinity was lost. They concluded that the O157 antigen somehow interfered with *E.* coli O157:H7 binding to epithelial cells; the reason for this is unclear.

1.13.3 Antibody responses to LPS

A number of studies have examined the sera of patients convalescing from *E. coli* O157 infection for antibodies to *E. coli* O157 LPS. In all cases most of the sera examined were found to contain antibodies to O157-LPS (Chart *et al* 1989, Greatorex and Thorne, 1994). As mentioned previously the O-polysaccharide is exposed at the bacterial surface and the above studies show that the LPS is indeed immunogenic. Polysaccharide antigens often

generate IgM responses only, and the study of Chart *et al*, (1989) showed that most of the antibodies they detected were of the IgM class. However LPS is often associated with large amounts of protein, this may facilitate the generation of an IgG response. Greatorex and Thorne (1994) reported both IgM and IgG responses in the sera they examined. The response to whole LPS must however be considered as the sum of the responses to both the core and the O-antigen (Currie *et al*, 2001).

Currie *et al* (2001) used an inhibition ELISA to determine exactly how much of the response to *E. coli* O157 LPS was due to the core component. When compared with the sera from convalescent patients the inhibitory effect of the R3 core was greatest among the healthy controls. This suggested that in health most of the response is directed towards the core LPS, and in disease, exposure to the O-antigen generates a significant response. A number of serotypes, for example O55, O91, O15, O86 and O157 (Amor *et al*, 2000, Currie and Poxton, 1999, Gibb *et al* 1992) are known to carry R3 core LPS. There are also many shared epitopes among all *E.* coli core types as well as with the cores of LPS from other organisms, for example *Salmonella* and *Shigella* (Currie *et al*, 2001). It is therefore likely that healthy humans exposed to specifically R3 core LPS and core LPS of all types, generate an anti-core LPS response that is largely cross-reactive with all five *E.* coli core types and detectable at any time.

Whole gut lavage (WGL) with a non-absorbable cleansing fluid can be used to detect IgA secreted into the gut. Currie *et al* (2001) used this technique to

measure the IgA response to the O157 antigen and the R3 core. They reported that among convalescent patients who went on to make a full recovery with no complications, the level of O157 and R3 specific IgA was significantly higher than in the healthy volunteers. The generation of the mucosal response was a possible factor in the recovery of these patients and may have helped prevent the development of serious complications. Hoque *et al* (2000) utilised the same technique to compare the mucosal immune responses of healthy volunteers from Edinburgh, UK and Dhaka, Bangladesh to core LPS. They reported that the level of endotoxin incomplete-core antibody (EndoCAb) was significantly higher among the group from Dhaka. It is thought that increased exposure to core LPS in the developing world may induce greater mucosal immunity and explain reduced levels of gastrointestinal illness and disorders in this part of the world (Hoque *et al*, 2000).

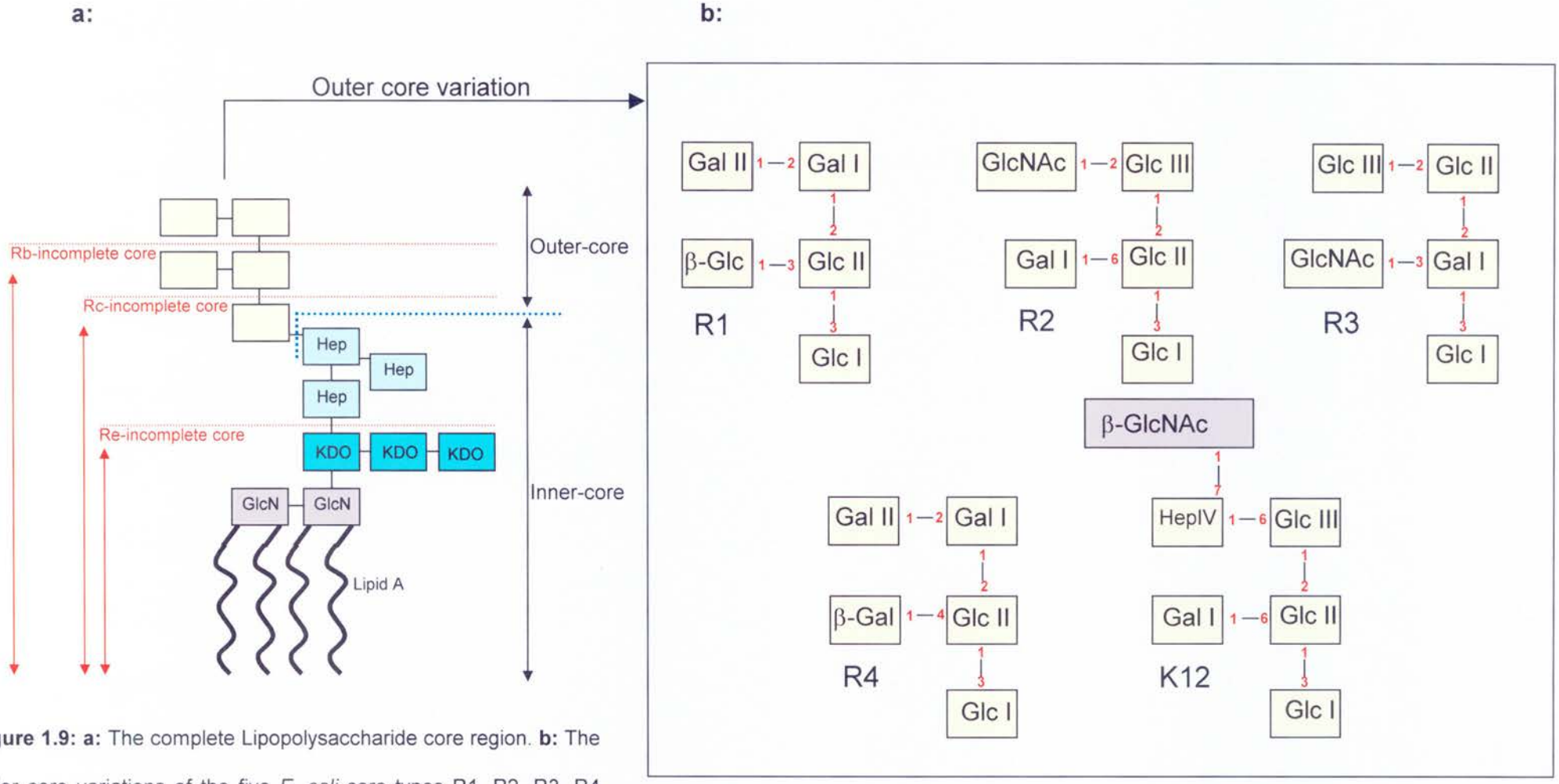


Figure 1.9: a: The complete Lipopolysaccharide core region. **b:** The outer core variations of the five *E. coli* core types R1, R2, R3, R4 and K12.

1.14 Antibody responses to *E. coli* O157

1.14.1 Early findings

A number of studies have examined the immune response to surface and secreted components of *E. coli* O157:H7. While most of these studies have focused on the human response to EHEC/EPEC infection, animal models have been used to examine the potential for vaccine development. Studies of this nature allow us to understand which antigens are recognised by the immune system and therefore may help identify potential vaccine targets. Early evidence for responses to EPEC infection was obtained by Levine *et al* (1985). While investigating the effects of a 50-70Mda plasmid on EPEC infection in human volunteers, a 94KDa protein unique to EPEC, was found to elicit both serum IgA and IgG responses. These findings were confirmed by Frankel *et al* (1996). They suggested that the 94KDa protein was most likely intimin. Mice challenged with the organism responsible for murine colonic hyperplasia, *Citrobacter rodentium*, which contains a pathogenicity island homologous to the LEE, were found to mount IgA responses to both intimin and EspB. These antibodies were predominant among those mice that survived the challenge and were therefore implicated in protection against infection. Lissner *et al* (1996) used a colostral immunoglobulin preparation (lactobin®) to examine its potential as a therapeutic agent for the treatment of EHEC disease. Antibodies reactive to EHEC haemolysin and verotoxin 1 and 2 B subunits [albeit truncated Vt1/2-GST fragments as prepared by Gunzer and Karch, (1993)] were detected in the colostral preparations. The study went on to show that these antibodies were

functional and were able to neutralise the Vero cytotoxic effects of the verotoxin and also the haemolytic activity of the haemolysin. The studies by Frankel *et al* (1996) and Lissner *et al* (1996) showed that antigens involved in the pathogenesis of EPEC/EHEC disease were antigenic and could give rise to functional antibodies.

1.14.2 Experimental EPEC infection in humans

The first study to investigate the role of pre-existing antibody in protection against EPEC disease was conducted by Donnenberg *et al* (1998). Two experiments looked at the ability of human experimental EPEC challenge to confer protection against a subsequent rechallenge with both heterologous and homologous strains. The heterologous challenge comprised an initial infection of 17 human volunteers with EPEC strain O55:H6, followed 27 days later by rechallenge of eight of the original 17 volunteers along with a further six naïve individuals, with EPEC strain O127:H6. In the second study, 13 (plus nine naïve individuals) volunteers received either an initial challenge with EPEC O127:H6 or an *eae* mutant of the same strain, followed 70 days later by a rechallenge with EPEC O127:H6. The results obtained failed to provide evidence for the generation of a protective antibody response. There was no significant difference in the incidence or severity of diarrhoea between any of the groups. It is possible that oral challenges of $1x10^9$ or $9x10^8$ cfu *E. coli* are too large to assess the effectiveness of any antibody response.

It is clear that intimin is a crucial component in the formation of AE lesions, thus an antibody reactive to intimin could prevent the formation of AE lesions. The existence of a number of intimin types (α, β, γ, δ, ε) (Adu-Bobie *et al*, 1998) may provide EHEC/EPEC organisms with the ability to avoid the neutralising capacity of these antibodies. Voss *et al* (1998) provided the first evidence for an immune response specific to an intimin type. They found that while patients convalescing from *E. coli* O111:H- infection reacted well to intimin from that particular strain, no reaction to the intimin of *E. coli* O157:H7 EDL933 was noted. Perna *et al* (1998) established that the *eae* sequences of *E.* coli O157 EDL933 and EPEC O126:H6 were 87.23% similar. The strains used by Voss *et al* (1996) showed a similar degree of *eae* homology (88.6%). Further analysis by Voss *et* al (1996) revealed that the terminal 200 amino acids of intimin contributed most of the variation seen in the whole protein. Since only a small region intimin appears to be variable it is unclear why Voss *et al* (1996) were unable to detect antibody responses to the large highly conserved N-terminal region of intimin.

To compensate for the variation in the Tir binding domain of intimin, Paton *et al* (1998) recognised distinct variations in Tir from different EPEC and EHEC strains. This was confirmed at the DNA level by Perna *et al* (1998) who identified significant divergences in *tir* sequences from the LEE of an EPEC and an EHEC strain. While convalescent sera from patients with HUS investigated by Paton *et al* (1998) reacted with all the Tir types tested, it is

unclear if these responses were to the conserved regions of the molecule or whether, as with the Tir binding domain of intimin, specific neutralising antibodies exist.

Studies by Li *et al* (2000) and Jenkins *et al* (2000) used recombinant antigens to investigate further the human response to EHEC infection. Upon admission to hospital sera from patients infected with *E. coli* O157 were collected, followed by convalescent samples eight and sixty days after admission. Li *et al* (2000) investigated these sera for antibodies reactive to GST-tagged EspA, intimin, Tir and EspB. In each case the response at day zero (sample obtained upon admission) showed little reactivity to any of the fusion proteins. However by day eight strong reactions to Tir, intimin, EspA and EspB were noted. These responses were still detectable 60 days after infection. Jenkins *et al* (2000) extended this study further by examining the response to three separate Tir domains; Tir-N (N-terminal region), Tir-M (region located between two transmembrane domains with intimin binding capacity) and Tir-C (C-terminal region),as well as Int280, EspB, EspA. They were able to detect antibodies reactive to all the recombinant antigens except Tir-N and Tir-C. The lack of any detectable antibody response to these two Tir domains may have resulted from the low homology between *tir* used in the generation of the recombinant C and N fragments and the *tir* from the strain infecting the patients tested (Jenkins *et al* 2000). It is also likely that due to the intracellular localisation of the Tir-N and C domains that their inaccessibility to the immune system resulted in the lack of any detectable

antibody. Although the intimin binding domain of Tir was strongly reactive with five of the eight patient sera tested it is possible that within this 24KDa Tir fragment that there is a smaller variable domain required to recognise and bind the five intimin types. In both studies by Li *et al* (2000) and Jenkins *et al* (2000) EspB generated the weakest response. This may be due to its initial role as an intracellular signalling activator within the host cell, followed by its eventual role as a pore at the end of the needle complex, which may render the protein inaccessible to the immune system.

1.14.3 Vaccine potential

A study by Ghaem-Maghami *et al* (2001) investigated the potential effectiveness of an intimin-based vaccine in murine colonic disease caused by *C. rodentium.* Mice challenged orally with *C. rodentium* were found to mount cross-reactive serum IgG and IgA responses against EPEC EspA, EspB and TirM (IgG only). *C. rodentium* expresses intimin β and as such an intimin β specific immune response was detected in these animals that did not cross react with EPEC intimin α. The study went on to try and establish whether or not acquired immunity to EPEC pathogenicity factors could induce protection as inferred by Donnenberg *et al* (1998). Two groups of mice were challenged either with wild type *C. rodentium.* Three months later convalescent mice and a group of naïve mice were rechallenged with either wild type *C.* rodentuium or an *eae* mutant of the same strain expressing EPEC intimin α via a plasmid. Convalescent mice were found to have significantly lower levels of infection than the naïve group. This demonstrated

that the development of acquired immunity was able to confer protection. While the results appeared to suggest that immunity to heterologous intimin types could result from a prior exposure to any of the five intimin types, it is likely that this arises from the large number of antibodies cross reactive to components involved in AE lesion formation. Thus anti-intimin antibodies are only part of the protective antibody repertoire. Indeed subsequent experiments by Ghaem-Maghami *et al* (2001) showed that immunisation with the carboxy terminal 280 amino acids of intimin α (Int280α) could protect mice from infection with *C. rodentium* expressing Int280α but did not confer protection against the wild type *C. rodentium*.

1.15 Verotoxin

1.15.1 Types

Karmali *et al* (1983) reported the vero cytotoxic activities of a toxin produced by *E. coli* isolated from the stools of patients with HUS. The toxin was named verotoxin. We now know that the toxin is phage encoded and is the cause of HC and HUS. O'Brien *et al* (1982) and subsequently Strockbine *et al* (1986) showed that antiserum raised against the shiga toxin (ST) of *Shigella dysenteriae* could neutralise the effects of the verotoxin. This led to the designation of the term 'shiga-like toxin' (SLT) still used today. Since then a number of SLT variants have been described. SLT 1 is identical to ST (Tesh *et al* 1993, Strockbine *et al*,1988). SLT 2 is antigenically distinct from SLT 1 but does share significant sequence homology with ST (Jackson, 1990). There are a number of recognised variants of SLT 2. SLT 2e is associated

with oedema in pigs (Valdivieso-Garcia *et al*, 1996 and Obrig, 1997). SLT 2c shares 99.6% and 94.8% homology with the structural components (A and B subunits) of SLT 2 (Furst *et al* 2000). Molecular typing techniques can be used to further divide SLT 2c into 2 groups known as SLT2vh-a and SLT2vh-b (Pierard *et al*, 1998). EHEC are known to produce one or two toxins, SLT1 or SLT2 (or both) (Mukherjee *et al*, 2002)

1.15.2 Structure

Verotoxin is a member of a group of toxins that share structural similarities. Like the toxins of *V. cholerae* and *S. dysenteriae*, the verotoxin produced by EHEC comprises a single active (A) subunit and five identical binding (B) subunits, denoted an AB_5 structure (**Figure 1.10**: Nataro & Kaper, 1998). The 32KDa A subunit is responsible for the activity of the toxin and is itself activated upon partial cleavage into subunits A_1 and A_2 that remain bound via a disulphide bond. The enzymatic activity is localised to the 27KDa A_1 subunit and the 4KDa A_2 subunit forms the site of attachment to the five B subunits (Nataro & Kaper, 1998). As previously mentioned the B subunits associate with one another to form a pentameric structure with a molecular mass of 38.5KDa, each individual subunit being 7.7KDa. This region is the toxin's receptor binding domain.

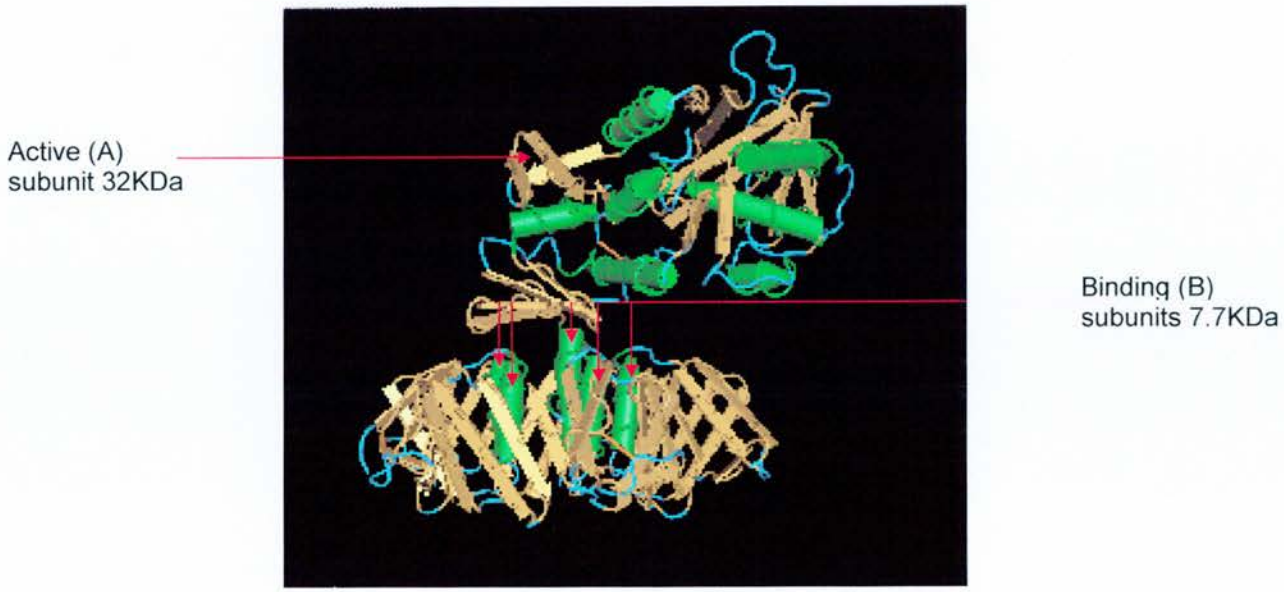


Figure 1.10: 70KDa Verotoxin, showing the classical AB_5 structure. The 32KDa active subunit and five 7.7KDa binding subunits are labelled

1.15.3 The verotoxin Receptor Gb_3

With the exception of Vt2e the receptor for verotoxin is the glycolipid globotriaosylceramide (Gb_3) (Vt2e:Gb_4). Gb_3 is known to occur in great abundance in the cortex of the human kidney, predominantly on the surface of cortical tubular epithelial cells (Lingwood, 1994 and Hughes *et al*, 1998). Indeed the levels of Gb_3 found on these cells are 50-100 fold higher than levels found on Vero cells a well established VT sensitive cell line (Lingwood, 1994). This is compelling evidence for the role of VT in the development of HUS. The B subunit of verotoxin is responsible for engaging and binding to the Gb_3 receptor thus allowing the A subunit access to the cell. Crystal structure analysis of the verotoxin B subunit revealed three potential binding

sites for Gb_3 on each monomer (Stein *et al*, 1992). Solution structure analysis conducted by Shimuzu *et al* (1998) subsequently revealed that only one of these binding sites was occupied by the Gb_3 receptor. The presence of multiple binding sites on the B subunit indicates that the process of binding to Gb_3 may be very complicated, it may also infer differential binding site usage by different VT types (Peter & Lingwood, 2000).

Lingwood (1994) conducted an extensive examination of both human adult and infant renal tissue for the presence of the Gb_3 receptor. Glomerular binding of VT1 in adult samples was not observed. However, there was clear binding of the toxin to proximal and distal tubules. In contrast, although paediatric samples showed decreased binding overall, in samples from infants less than two years old, all the glomeruli were found to bind VT1, however by two and a half years the level of binding had reduced to 50% of samples observed. The Gb_3 receptor has also been found in the gastrointestinal tract (Peter & Lingwood, 2000). These are important observations in terms of HUS as the apparent reduction in glomerular Gb_3 receptor expression at 2.5 years corresponds to the upper age limits of HUS (Rowe *et al*, 1991). The observed reduction in Gb_3 expression in the adult glomerulus appears not to correspond with the frequency of HUS among elderly adults.

1.15.4 Entry into the cell

Once bound the toxin must enter the cell in order to exert its actions. The entry of verotoxin into cells has been the subject of much work and will be described briefly here. Verotoxin enters cells via receptor mediated endocytosis (Sandvig *et al*, 1992). The presence of Gb_3 on a cell surface does not however predict that that cell will be sensitive to verotoxin. Factors other than Gb_3 binding are required for toxicity. A number of cells (for example epidermoid carcinoma A431 cells) are known to bind and internalise VT and are yet unaffected (Johannes & Goud 1998). Shiga toxin is internalised via clathrin coated pits and is then transported to the Golgi apparatus (Sandvig *et al*, 1992 and Johannes & Goud 1998). It is then thought that the verotoxin is released from the Golgi into the cytoplasm.

1.15.5 Mode of action

Verotoxin is a potent inhibitor of protein synthesis able to inactivate 60 S ribosomal subunits selectively (Obrig *et al*, 1987 and O'Brien *et al*, 1992). The ability of shiga toxin to inhibit protein synthesis in rabbit reticulocyte lysates was noticed by Brown *et al* (1980). Furthermore they established that this inhibitory effect could be enhanced by the addition of proteolytic enzymes and reducing agents. Proteolytic digestion would have released the A_1 fragment which has potent protein synthesis inhibition activity.

Shiga toxin causes ribosomal inactivation by preventing interaction with elongation factors-1 and 2 (EF-1 and EF-2) and therefore arrests protein

translation at the aminoacyl-tRNA binding step (Obrig *et al*, 1987, Endo *et al*, 1988). Obrig *et al* (1987) investigated the specifics of EF-1 and 2 inhibition by Shiga toxin. They found that shiga toxin prevented the enzymatic binding of radiolabelled tRNA to ribosomes obtained from rabbit reticulocytes. This is achieved via the N-glycosidase activity of the A_1 subunit which removes an adenine residue from the 28 S rRNA of the 60 S ribosomal subunit, thus preventing interaction with EL-1 and EL-2 (O'Brien *et al*, 1992).

1.16 Disease in cattle

It is widely accepted that carriage of *E. coli* O157 by cattle leads to outbreaks of human disease. Despite this however, adult cattle remain unaffected by this particular serotype (Cray and Moon, 1995) while infection in calves usually results in mild diarrhoea (Wales *et al*, 2001). The VTEC O26 has been associated with both human and bovine disease (Gunning *et al*, 2001). In humans disease symptoms often include diarrhoea, bloody diarrhoea, HC and HUS while in calves, *E. coli* O26 causes diarrhoea (Gunning *et al*, 2001). Examination of post-mortem tissue samples from the intestines of two calves naturally colonised with *E. coli* O26 revealed the presence of AE lesions (Gunning *et al*, 2001). In another case, *E. coli* O26 were found intimately attached via AE lesions in the colon of an eight-month old heifer with bloody diarrhoea (Pearson *et al*, 1999). The reason for the lack of *E. coli* O157 associated disease in adult cattle is unclear, although the expression of variant intimin types may play a role.

Studies to examine the susceptibility of various bovine tissues have revealed interesting results. Studies by Dean-Nystrom *et al* (1998) demonstrated the ability of *E. coli* O157:H7 to produce AE lesions *in vivo*. It is important to realise however that animals used in these experiments were neonatal colostrum deprived calves. As such they are susceptible to infection by *E. coli* O157. It does prove however that bovine ileal cell types are not intrinsically resistant to AE lesion formation.

These findings were expanded upon by Baehler *et al* (2000). They focused on the ability of *E. coli* O157 to produce AE lesions in adult bovine rectal and colonic tissue explants. After 6-9h incubation periods the tissue explants were examined by electron microscopy for the presence of AE lesions. Lesions were observed on all inoculated cells along with associated destruction of the microvillus ultrastructure. AE lesion formation on the surface of the bovine tissue explants was comparable to the prolific formation of AE lesions seen on HeLa cell monolayers. It was clear therefore that AE lesion formation was as likely to occur on adult tissue as it was on neonatal tissue. The work also showed that the bovine ileum, colon, caecum and rectum, were all susceptible to AE lesion formation. The results presented by Dean-Nystrom *et al* (1998) are highly suggestive that the bovine immune system plays an important role in the protection against EHEC disease.

The role (if any) verotoxin plays in bovine EHEC colonisation is unclear. Toxin expression is known to occur *in vivo* and has been detected in the faeces of colonised animals (Donkersgoed *et al*, 1999). A number of studies have used PCR/DNA probes to determine which toxin type(s) a bovine isolate carries. Blanco *et al* (1996), China *et al* (1996), Chapman *et al* (1997) and Orden *et al* (1998) have all demonstrated the prevalence of VTEC among cattle populations.

A study by Pruimboom-Brees *et al* (2000) demonstrated that tissue from the gastrointestinal tract of newborn calves was devoid of the verotoxin receptor,

Gb_3. Gb_3 expression was observed in the kidney and brain however. Further more, ligated ileal loop experiments in calves revealed that *E. coli* O157:H7 was not enterotoxic. The lack of gastrointestinal Gb_3 was thought to explain the apparent resistance to the effects of verotoxin demonstrated by cattle. Hoey *et al* (2002) expanded on this study, by examining rumen, jejunum, ileum, caecum, colon and kidney tissue samples from one and ten month old cattle. VT1 was found to bind to the crypt cells adjacent to the submucosa from the jejunum, ileum, caecum and colon samples from both age groups. Gb_3 was successfully extracted from the mucosal tissue samples confirming the presence of verotoxin receptors in bovine gastrointestinal tissue. The pattern of Gb_3 receptor distribution in bovine kidney (collecting ducts and tubules) was similar to that observed by Lingwood (1994) in human renal tissue. These findings were contradictory to those of Pruimboom-Brees *et al* (2000). In contrast to human endothelial cells that have previously been shown to possess Gb_3 and bind verotoxin (Jacewicz *et al*, 1999), no verotoxin binding to bovine endothelial cells was reported by either Hoey *et al* (2002) or Pruimboom-Brees *et al* (2000). This difference in Gb_3 expression may explain the verotoxin susceptibility differences between humans and cattle.

Recent evidence has begun to suggest that cattle colonisation by *E. coli* O157:H7 may be directed to a specific tissue type. Grauke *et al* (2002) carried out extensive post-mortem investigations of sheep and cattle experimentally infected with *E. coli* O157 to determine the gastrointestinal

location of *E. coli* O157 colonisation. Tissue and digesta samples were taken from the rumen, abomasum, duodenum, lower ileum, caecum, ascending/descending colon and rectum of experimentally infected sheep at weekly intervals up to 43 days post infection. *E. coli* O157 were predominantly isolated from the digesta of the lower gastrointestinal tract, and no *E. coli* O157 were isolated from any of the tissue samples taken. Similar results were seen in the experimentally infected cattle. Rumen and duodenum digesta samples showed that beyond 16 days post infection no *E. coli* O157 could be isolated from the samples collected. However up to 34 days post infection *E. coli* O157 could be detected in the faeces of the infected animals. This was highly suggestive of the fact that *E. coli* O157 may preferentially localise to the lower regions of the gastrointestinal tract. Recently Naylor *et al* (unpublished data) have been able to characterise further the region of *E. coli* O157 colonisation in both experimentally infected and naturally colonised animals. Their data provide support for the findings of Grauke *et al* (2002) and have narrowed the colonisation region to an area of stratified squamous epithelium unique to the terminal rectum. This tissue appears to persist for only a number of centimetres, but levels of *E. coli* O157 isolated from here far exceed levels elsewhere in the bovine digestive tract. Whether or not this phenomena is the result of tissue tropism exhibited by *E. coli* O157 is yet to be clarified. If confirmed this may have important implications for our understanding of how *E. coli* O157 interact with the bovine host.

Hypothesis and aims of this thesis

It is unclear what role (if any) the bovine immune response plays in protection against EHEC associated disease. It is possible that antibody responses mounted against a number of antigens present on *E. coli* O157 offer a degree of protection. It is hypothesised that cattle are exposed to R3 core LPS more frequently than humans and consequently develop an immune response against this LPS core type that could afford protection against EHEC infection. It is also hypothesised that this increased exposure to R3 core LPS also leads to an increase in exposure to antigens encoded upon the LEE pathogenicity island from which an antibody response may develop.

The introduction to this thesis cited a number of factors, shown to be immunogenic in other species, which are worthy of investigation. LPS is the dominant antigen of the Gram-negative outer membrane and as such is likely a very important component of any immune response. Studies have proved an association between R3 core LPS and the VTEC virotype and a proportion of bovine VTEC isolates have also been shown to harbour the LEE pathogenicity island. The frequency with which R3 core LPS appears among the bovine commensal *E. coli* flora may influence the level of response-mounted against this antigen.

The LEE pathogenicity island presents the bovine host with an array of antigens against which an immune response may be mounted with the ultimate aim of preventing intimate adherence. However it is unclear which LEE antigens cattle are exposed to and whether or not detectable immune responses result from exposure. It is likely though that a number of the LEE

components are immunogenic in cattle and that these antibodies have the potential to block intimate adherence.

Therefore the aims of this thesis were as follows:

1) To determine the distribution of the five LPS core types of *E. coli* within two populations of faecal *E. coli* isolates from healthy humans and cattle.
2) To examine sera from healthy adult human volunteers and healthy adult cattle for antibodies reactive to each of the five core types and to investigate a link between core LPS exposure and anti-core LPS antibody levels.
3) To clone, express and purify recombinant intimin280 (Int280), *E. coli* secreted protein B (EspB) and Verotoxin 2 B subunit (Vt2B).
4) To use Immunoblot and ELISA to detect antibodies to recombinant Int280, EspB and Vt2B and LPS in the sera and faecal antibody preparations of calves experimentally challenged with *E. coli* O157.
5) To determine whether any antibody detected in the sera of experimentally infected calves inhibits the formation of attaching effacing lesions produced by *E. coli* O157.

Chapter Two

Materials and Methods

2.1 Bacterial Strains

All *Escherichia coli* strains used in this study were supplied by the Zap Lab, Medical Microbiology, The University of Edinburgh unless otherwise stated. **Table 2.1** summarises the strains used.

Table 2.1: Strains used throughout this study

Technique (section reference)	*Escherichia coli* strain
PCR (section 2.2)	Zap 26, EDL933 Sequenced strain
Competent cell preparation and Cloning (section 2.3 & 2.6)	*E. coli* AECC 185
Outer membrane preparations (section 2.16)	Zap 193 (NCTC 12900) Verotoxin Negative
Experimental Infections (section 2.22)	Zap 3 Lothian Red House dairy outbreak strain (cattle isolate)
	*Zap 198 Walla 3, outbreak strain Verotoxin Negative
	*Zap 196 Walla 1, outbreak strain Verotoxin Positive

* Walla strains originated from food poisoning outbreaks in Washington State. Walla 3 is identical to Walla 1 but has lost the verotoxin 2 encoding phage and does not carry any other verotoxin genes

Dr Fiona Thomson-Carter of The University of Aberdeen and Dr Michael Pearce of the Wellcome Trust IPRAVE study provided healthy adult bovine commensal *E. coli* isolates for use in the LPS core typing studies (**section 2.2**). Isolates represented cattle from around Scotland. Human commensal *E. coli* isolates were obtained from 197 healthy volunteers aged approximately 20yrs at the University of Edinburgh. All isolates were sub-cultured on Columbia agar supplemented with horse blood overnight at 37°C. Individual colonies were then transferred to nutrient agar slopes, after overnight incubation at 37°C isolates were stored at 4°C until required.

2.2 PCR

2.2.1 DNA Template preparation

All recombinant antigens were generated by primary PCR from *E. coli* O157:H7 EDL933 (**Table 2.1**). Overnight 5ml cultures were grown in Luria Bertani (LB: 10g peptone from casein, 5g Yeast extract, 10g Sodium chloride, Merck Darmstadt, Germany) broth at 37°C in containment level 3 with shaking. Volumes (10μl) of the overnight cultures were used to inoculate 100μl sterile ultra pure water (Sigma-Aldrich Company Ltd, Poole, Dorset, UK). Subsequent heating at 100°C for 10 min ensured complete lysis of all the bacteria. The resultant lysate formed the template from which all reactions proceeded. Other *E. coli* were sub-cultured on blood agar prior to lysate preparation. DNA templates for the determination of LPS core type by PCR were obtained as described above.

2.2.2 Primers and reaction volumes

Primers used for the generation of recombinant Int280 and EspB were supplied by the Zap lab, Medical Microbiology, The University of Edinburgh. Dr David Gally of the Zap lab designed primers for the amplification of the verotoxin B subunit gene from the *E. coli* EDL933 sequence.

Primers for the determination of LPS core type were taken from published sequences (Amor *et al* 2000; see **Table 2.2** for details). Multiplex PCR was not carried out. All isolates were initially screened for the presence of R1 core specific genes. Those failing to generate any products were re-screened in turn for R2, R3, R4 and K12 until each had been assigned a core type. Any that remained unclassified were labelled as 'untypeable'

All reactions were done in a final volume of 50μl. **Table 2.2** shows the primer sequences and cycling conditions used in each reaction.

Each reaction mix contained the following:

10x PCR buffer (Roche)	5μl
dNTPs: 1.25mM: (Amersham)	2μl
Primers: (50pM): (MWGbiotech)	1μl
Taq DNA polymerase: 5Uμl^{-1}: (Roche)	0.15μl
Template	5μl
H_2O	36.85μl

Table 2.2. Primer sequences and reaction conditions used to produce recombinant proteins and determine LPS core types. Primers 1 to 6 were designed to amplify both antigens from the LEE and the verotoxin B subunit. Primers 7 to 16 were used to ascertain the LPS core type of commensal *E. coli* isolates.

	Primer	Sequence 5'- 3'	Initial denaturation	Cycle conditions	Final extension
1	eaeA280sense	GGG GAT CCG CCA GCA TTA CTG AGA TTA AG	94°C 4 min	35 cycles 94°C 40 s 54°C 45 s 72°C 1 min 30 s	72°C 2 min
2	eaeAantisense	GGG GAT CCA TGA TTA CTC ATG GTT GTT ATA CC			
3	espBsense	GGG GAT CCA CGA TGG TTA ATT CCG CTT CGG			
4	espBantisense	GGG GAT CCC CAG CTA AGC GAC CCG			
5	Vt2Bsense	CCG GAT CCA TGA AGA AGA TGT TTA TGG CGG			
6	Vt2Bantisense	CCG GAT CCC ACT TGT TAC CCA CAT ACC AC			
7	R1C3	GGG ATG CGA ACA GAA TTA GT	94°C 4 min	35 cycles 94°C 20 s 50°C 30 s 72°C 2min 15 s	72°C 2 min
8	R1K15	TTCCTGGCAAGAGAGATAAG			
9	R2C4	GAT CGA CGC CGG AAT TTT TT			
10	R2K9	AGC TCC ATC ATC AAG TGA GA			
11	R3C2	GGC CAA AAC ACT ATC TCT CA			
12	R3K13	GTG CCT AGT TTA TAC TTG AA			
13	R4C4	TGC CAT ACT TTA TTC ATC A			
14	R4K14	TGG AAT GAT GTG GCG TTT AT			
15	K12-1	TTC GCC ATT TCG TGC TAC TT			
16	K12-2a	TAA TGA TAA TTG GAA TGC TGC			

Primers 1 and 2 used for the amplification of the Intimin gene *eae*. Primers 3 and 4 were used to amplify the EspB gene *espB* and 5 and 6 were used to amplify the gene encoding the verotoxin B subunit. Primers 7-14 were used to detect genes involved in LPS core type biosynthesis, the first two characters in the primer suffix denote the core type specific genes detected

2.3 Determination of LPS core type by dot blot.

E. coli were sub-cultured overnight at 37°C on Columbia agar supplemented with horse blood. Single colonies were emulsified in 100μl of water. Nitrocellulose membranes were cut and marked into grids with squares of dimensions 10mm x 10mm. The nitrocellulose grids were washed in Tris buffered saline (TBS: 0.02M Tris-HCl, 0.5M NaCl pH7.5) and allowed to dry at 37°C. Volumes (5μl) of each emulsified bacteria solution were applied to each of the squares on the nitrocellulose membrane. Controls comprising heat killed cells and PCP extracted core LPS representing core types R1, R2 and R3 (**section 2.19**) were included on each nitrocellulose grid. Nitrocellulose was incubated at 37°C to dry. Membranes were blocked with 3% fish gelatin (v/v) in phosphate buffered saline (PBS pH7.4: Oxoid) for 45 min at room temperature. Monoclonal antibodies as described by Gibb *et al* (1992) were used for the determination of LPS core type. Monoclonal antibodies were diluted 1 in 50 in 1% fish gelatin + PBS (pH7.4) prior to use. Nitrocellulose membranes were incubated in 30 ml of the appropriate monoclonal antibody solution for 3h at room temperature. Membranes were then washed twice in tween-Tris buffered saline (TTBS: 0.02M Tris-HCl, 0.5M NaCl, 0.025% tween 20 pH7.5) for 10 min. Rabbit anti-mouse IgG horse radish peroxidase (HRP: Sigma) was diluted 1 in 1000 in 1% fish gelatin + PBS and incubated with the nitrocellulose membranes for 1h 30 min. Membranes were washed twice in TTBS for 10 min, rinsed in H_2O and developed in HRP colour reagent (Bio-Rad, UK)

2.4 Competent cell preparation

A single colony of *E. coli* AECC 185 was used to inoculate 20ml LB broth in a 250ml flask. The culture was grown at 37°C with shaking until the optical density at 600nm (A_{600}) was 0.5. The 20ml culture was then diluted in 100ml of LB broth and growth continued until A_{600}=0.5. Finally 400ml of LB broth was added to the culture and growth continued until A_{600}=0.6. The flask was placed immediately on ice. Bacteria were harvested by centrifugation at 4000g, 4°C for 10min. Cells were resuspended in 100ml ice cold sterile TFB1 (30mM KAc, 10mM $CaCl_2.2H_2O$, 100mM KCl, 15% glycerol v/v, 50mM $MnCl_2$) and bacteria were harvested by centrifugation at 4,000g, 4°C for 10min. Harvested bacteria were resuspended in 20ml sterile ice cold TFBII (75mM $CaCl_2$, 10mM KCl, 15% glycerol v/v, 100mM Na-MOPS pH7). Aliquots (100μl) were frozen in liquid nitrogen and stored at -70°C until required.

2.5 Agarose gel electrophoresis

PCR products and plasmids were resolved by agarose gel electrophoresis. Gels were prepared by the addition of 1g agarose to 100ml Tris-acetic acid-EDTA (TAE) buffer (40mM Tris-acetic acid pH8, 1mM EDTA). Each gel was supplemented with 2nM (1.5μl at 10mgml^{-1}) ethidium bromide to allow visualisation of the DNA. Samples comprising 2μl of the PCR product along with 2μl gel loading solution (Sigma) and 6μl ultra pure water (Sigma) were loaded on to each gel. Products were run along side 2μl ready load 100bp ladder (Invitrogen Life sciences, Paisley, UK) in order to achieve accurate

sizing. Plasmid samples were prepared the same way and run along side the appropriate marker to allow either sizing (1Kbp ladder) or quantification (λHindIII).

A voltage of 100V was applied until the products had achieved sufficient resolution. Products were visualised under UV illumination and analysed by Bio-Rad Quantity One software.

2.6 PCR and restriction product clean-up

Each successful PCR or restriction digest reaction was subjected to a QIAquick® clean-up column (Qiagen, Crawley, W. Sussex, UK). Multiple reactions of the same PCR were pooled up to the maximum capacity of the column, cleaned, and then eluted in the required volume of elution solution (30 or 50μl:10mM Tris-HCl, pH8.5). Product recovery from the column was determined by agarose electrophoresis.

2.7 Transformation

Chemically competent *E. coli* AECC 185 were transformed by the following method. Purified plasmid (5μl) was added to 100μl chemically competent cells for 30 min on ice. Bacteria were then heat shocked at 42°C for 30 s. Immediately 1ml LB broth was added and the bacteria were incubated at 37°C for a further 2 hours with gentle shaking. Luria Bertani agar (Merck) supplemented with 1.3μM ampicillin (LBamp) was inoculated with 100μl of the competent cell culture. Plates were incubated overnight at 37°C.

2.8 Plasmid preparation.

After transformation approximately 10 colonies from the LBamp plates were sub-cultured overnight at 37°C with shaking in 5ml LBamp broth. Bacteria were harvested in a bench centrifuge at 3000g for 15 min. The supernate was discarded. Plasmids were extracted using QIAprep® kits (Qiagen). In each case the optional wash was performed and prior to plasmid elution, the columns were incubated at 42°C for 5 min to ensure removal of residual ethanol. Plasmids were eluted in 30μl of Qiagen elution solution. Plasmids were visualised by agarose gel electrophoresis and ethidium bromide staining as described in **section 2.5**.

2.9 Restriction digests

All plasmid DNA digests were carried out in a maximum 10μl reaction unless otherwise stated. To 2μl of extracted plasmid DNA, 20U of restriction enzyme, 1μl 10x buffer as supplied by the manufacturer and autoclaved ultra pure water (Sigma) to a final volume of 10μl were added. The reaction was allowed to proceed in a water bath at 37°C for 1 hour. The samples were then removed, gently mixed, and pulsed in a microfuge and replaced in the water bath for a further hour. Enzymatic reactions were cleaned as described in **section 2.6**.

2.10 TOPO pCR®4 cloning

To ensure the primers had engineered functional *Bam*H1 restriction sites, PCR products were cloned into pCR®4 TOPO vectors (TOPO TA cloning kit,

Invitrogen). Transformation and plasmid preparation were carried out as described previously. Restriction enzyme, BamH1, was used to excise the cloned fragment from the pCR®4 TOPO vector. The restriction products were resolved by agarose electrophoresis (**section 2.5**).

2.11 Gel purification

Cloned fragments were extracted from plasmid DNA by large-scale restriction digests and subsequent purification of the excised fragment directly from the gel. Volumes of purified plasmid (20μl) were added to 60U restriction enzyme along with 3μl of the recommended buffer (10x) and H_2O to a final volume of 30μl. The reaction was allowed to proceed as described in section 2.8. Products were resolved by agarose gel electrophoresis. To allow better separation of the restriction fragments a 0.8% gel (0.8g agarose in 100ml TAE buffer) was prepared. A UV trans-illuminator was used to visualise the DNA bands in the gel. The BamH1 restricted cloned PCR product was cut from the gel. The agarose piece was then placed in a QIAquick® column (Qiagen) and heated to 65°C in a water bath to melt the agarose. Once melted the Qiagen clean up protocol was used to purify the fragment. The fragment was eluted in 30μl Qiagen elution solution.

2.12 pGEX-4t_2 preparation and restriction

Chemically competent *E. coli* AECC 185 were transformed with the commercial cloning vector pGEX-4t_2 (Amersham Pharmacia Biotech, UK) as described previously (**section 2.7**). Transformed colonies were sub-cultured

overnight at 37°C in 5ml LB broth supplemented with ampicillin (as described in **section 2.7**). The plasmid was removed with a QIAprep® kit (Qiagen). The plasmid was digested with the appropriate enzymes. The restricted, cleaned and purified plasmid was then treated with 3U shrimp alkaline phosphatase (SAP; Boehringer Mannheim) for 20 min to remove phosphate groups at the digested ends of the DNA. The enzyme was denatured by incubation at 65°C for 15 min.

2.13 DNA quantification and ligation

In order to ligate the gel-purified PCR products to the restricted, SAP treated pGEX-4t_2 vector, an approximate value for the amount of DNA present had to be calculated. This was achieved by resolving the restricted vector and all the PCR products by agarose gel electrophoresis alongside a DNA marker of known quantity. In this case the marker used was ready-load λHindIII (Invitrogen) (**Figure 2.1**). The amount of DNA present in each band is detailed in **Table 2.3.** The quantity of the DNA present was assessed by comparing the intensity of the sample bands to those in the marker. Each sample's intensity on the gel was compared to the band closest in size to the marker.

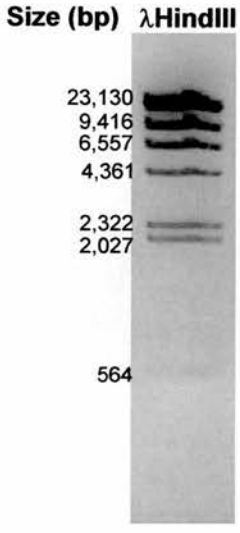


Figure 2.1: the λHindIII marker

Table 2.3: λHindIII marker quantification

λHindIII marker band size (bp)	Quantity of DNA ($ng2\mu l^{-1}$)
23, 130	95
9,416	39
6,557	27
4,361	18
2,322	9.6
2,027	8.4
564	2
125	0.5

Ready-load λHindIII marker (Invitrogen) contains $0.1\mu g\mu l^{-1}$DNA. The quantity of DNA in each band is given per standard $2\mu l$ application

To ensure efficient ligation three different molar vector : insert DNA ratios, 1:1, 1:3 and 3:1 were used. Using the figures obtained from the λHindIII ladder the following calculation showed how much DNA was to be used in each reaction.

$$\frac{\text{ng of vector x Kb size of insert}}{\text{Kb size of vector}} \times \text{molar ratio of } \frac{\text{Insert}}{\text{vector}} = \text{ng of insert}$$

Wherever possible the total reaction volume was 20μl. T4 ligase and ATP buffer (Invitrogen) were used as per the manufacturer's instructions. The ligation of the restricted PCR product to the cut vector was done overnight at 16°C in a Phoenix thermocycler (Helena BioSciences). Chemically competent AECC 185 *E. coli* were transformed with the ligate (section 2.6). After overnight incubation on LBamp agar at 37°C, colonies were examined for the presence of plasmid DNA with PCR product insert.

2.14 Orientation analysis

To ascertain the orientation of the inserted gene, restriction sites within both the vector and the inserted gene were used. **Table 2.4** shows the restriction enzyme used and the expected fragments in each orientation.

Table 2.4: Expected fragment lengths after restriction digest of three plasmids

Vector-gene	Restriction enzyme	Expected fragments correct orient. (bp)	Expected fragments incorrect orient. (bp)
pGEX-4t$_2$-Int280	Pst1	4000, 1880	4800, 1000
pGEX-4t$_2$-EspB	EcoR1	304, 5580	631, 5253
pGEX-4t$_2$-Vt$_2$Bsub	Hinf1	1213, 1184, 796, 517, 396, 338, 255, 191, 143, 75, 70, 55, 49	1213, 1050, 796, 517, 396, 338, 255, 191, 183, 143, 75, 70, 50

2.15 Sequence analysis

Clones identified to have a correctly orientated insert were sequenced. QIAprep® kits (Qiagen) were used as previously described, to produce plasmid preparations for each of the clones to be sequenced. Where the cloned fragment was longer than 500bp, the vector was sequenced in both forward and reverse directions to ensure that a sequence for the whole gene would be obtained. Sequencing was carried out by MWG-biotech (Germany).

2.16 SDS-PAGE

Protein solutions were mixed with an equal amount of double strength SDS-PAGE sample buffer and heated to 100°C in a boiling bath for 3 min. Proteins were resolved on 10% slab gels using the buffer system of Laemmli (1970), as described by Hancock and Poxton (1988). Details of all SDS-PAGE buffers are given in Appendix 1. LPS was resolved on 14% slab gels in the same buffer system without SDS in the gel buffers.

2.16.1 Coomassie Blue Staining

The proteins separated on SDS-PAGE gels were stained with Coomassie blue stain R250 (Fisher Scientific, UK) as described by Hancock and Poxton, 1988.

2.16.2 Zinc chloride staining

To allow immediate visualisation of proteins resolved by SDS-PAGE, gels were submerged in a 0.3M solution of $ZnCl_2$ for 5 min.

2.16.3 Immunoblotting

The Tris-glycine transfer method as described by Towbin *et al* (1979), was followed, (details of all immunoblotting buffers are given in Appendix 1). Proteins or LPS resolved by SDS–PAGE, were transferred to a nitrocellulose membrane (0.2μm pore size, Schleicher & Schuell, Germany) in Tris (0.025M), glycine (0.192M), methanol (20% v/v) buffer, pH 8.3 at 5V and 40 mA for 18 h at 4°C.

The following transfer procedures were carried out with gentle agitation. After washing the nitro-cellulose in Tris buffered saline (TBS: 0.02M Tris, 0.5M sodium chloride, pH 7.5) for 10 min the membrane was blocked with 15 ml of 3% (v/v) fish gelatin (Sigma) in TBS for 45 min. The nitrocellulose membrane was incubated with appropriate anti-sera diluted in 1% (v/v) fish gelatin in TBS. This was incubated at room temperature for 90min. For the detection of antibodies to LPS, the blots were left to incubate overnight at room temperature. After washing twice for 10 min in Tween TBS (0.02M Tris, 0.5M sodium chloride, 0.025% Tween 20, pH 7.5) the membrane was incubated with the appropriate antibody conjugate (HRP) diluted 1 in 2000 at room temperature for 90min. After two further washes in Tween TBS as above, the nitrocellulose membrane was washed three times in distilled water. The nitrocellulose was placed into HRP developer (details given in Appendix 1) and the colour developed. The development was stopped by several washes in distilled water.

2.17 Outer membrane preparations

Overnight 5ml cultures of Zap 193 (**Table 2.1**) were used to inoculate (0.1% v/v) 100ml LB broth. Cultures were grown at 37°C with shaking until A_{600}=0.8. Bacteria were harvested by centrifugation at 10,000g for 15min at 4°C, and washed in 50ml sterile PBS and harvested as before. The bacteria were resuspended in 9ml pyrogen free H_2O (pfH_2O). Bacteria were lysed by three rounds of sonication at 10μm for 30s with 30s rest. A volume (1ml) of a 7% sarkosyl (Sigma) solution was added at room temperature and clearing of the

opalescent solution observed. Cell debris was removed by centrifugation at 10,000g for 15min at 4°C. To harvest the outer membrane proteins the supernate was subjected to ultra-centrifugation at 50,000g for 1h at 4°C. The supernate was removed and the pelleted outer membrane proteins resuspended in 10ml pfH_2O, and stored at -20°C until required.

2.18 Secreted protein precipitation with trichloroacetic acid (TCA)

E.coli O157 Zap 193 (**Table 2.1**) was cultured overnight at 37°C on Columbia agar supplemented with horse blood (Oxoid). A single colony was used to inoculate 5ml sterile LB broth. The culture was incubated overnight at 37°C with shaking. Volumes (1ml) of the overnight culture were added to 40ml sterile minimal essential medium. The culture was incubated at 37°C with shaking until A_{600}=0.9. Volumes (20ml) of the culture were added to polypropylene universals. Bacteria were harvested by centrifugation at 4,000g for 15 min at room temperature. The supernate was removed and passed through a 0.2μm filter into a fresh polypropylene universal. TCA (100%) was added to a final concentration of 10% (v/v). Proteins were left at 4°C overnight to precipitate. Precipitated proteins were harvested by centrifugation at 4000g for 30 min at 4°C. The supernate was discarded and the pellet allowed to dry completely. Harvested proteins were resuspended in 500μl 1.5M Tris-HCl (pH8.0). Proteins were stored at -20°C until required.

2.19 LPS extraction by phenol chloroform petroleum and rapid aqueous phenol methods.

Phenol chloroform petroleum (PCP) extracted LPS cores R1, R2, R3, R4 and K12 were prepared by staff at the Microbial Pathogenicity Research Laboratorty, Medical Microbiology, The University of Edinburgh by the method of Galanos *et al* (1969).

Smooth LPS was extracted by the micromethod of Fomsgaard *et al* (1993). Briefly *E. coli* were cultured overnight at 37°C on Columbia agar supplemented with horse blood. Several colonies were emulsified in 500µl pfH_2O and subjected to the rapid aqueous phenol extraction protocol.

2.20 Production and purification of recombinant proteins

2.20.1 Protein induction

Overnight 5 ml cultures in LBamp of each clone were used to inoculate 1 litre LBamp. Cultures were grown to mid-log phase, as determined by absorption at 600nm, at 37°C with vigorous shaking (approx 4h). Isopropyl β-D-thiogalactoside (IPTG; Fisher) to a final concentration of 1mM was added to each flask and growth at 37°C (with shaking) was continued for a further 3 h, during which time protein production was induced. Bacteria were harvested by centrifugation at 10,000g for 15 min.

2.20.2 Protein purification and extraction.

Harvested bacteria were re-suspended in ice cold PBS. Bacteria were lysed by three rounds of sonication at 10μm for 30 s with 30 s rest. Cell debris was separated from the solution by centrifugation at 7,000g for 15 min. A sample of the supernate and harvested cell debris were removed and subjected to SDS PAGE (**section 2.15**). If the GST tagged protein was present in the supernate then it was stored at –20°C until required. If the fusion proteins had formed inclusion bodies, then further treatment was required to solubilise them.

2.20.3 Urea denaturation of insoluble inclusion bodies.

Harvested cell debris was re-solubilised in 8M urea supplemented with 1mM dithiothritol (DTT), insoluble debris was removed by centrifugation at 7,000g for 15 min. The supernate was then transferred to 12-14,000 molecular weight cut-off (mwco) dialysis tubing. The supernate was then progressively dialysed against reduced concentrations of urea (6M, 4M, 2M, 1M) for 2 h at a time. Each urea solution was supplemented with 1 mM DTT. Final dialysis against PBS + 1mM DTT was used to remove the urea completely; this was done for a further 2 h and then repeated. Precipitated proteins were removed by centrifugation at 70,000g for 30 min. The supernate was removed and stored at -20°C until required.

2.20.4 Affinity chromatography.

To remove specifically the GST tagged proteins from the supernate, GSTrap FF 1ml columns (Amersham) were used. The supernate was pumped on to the columns using a peristaltic pump according to the manufacturer's recommendations. Each column was equilibrated with five bed volumes PBS and then the sample applied to bind the GST tagged proteins. The column was again washed with PBS before the GST tagged proteins were eluted with ten bed volumes 10mM reduced glutathione (Sigma) plus 50mM Tris, pH8. Columns were washed with 6M guanidine HCl and 70% ethanol, and stored at 4°C in 20% ethanol. SDS-PAGE was used to confirm that the GST tagged proteins had been eluted from the column.

2.20.5 Protein Assay.

The level of protein in the pooled fractions from the GSTrap FF columns was determined by a modification of the method of Lowry (Lowry *et* al, 1951) with the following alterations. To reduce the amount of sample required for the assay, the protocol was scaled down so that the procedure could be performed on a microtitre plate. A bovine serum albumin standard curve was used to calibrate the assay. Volumes (40μl) of each of the standards were added in duplicate to the wells of a microtitre plate. The sample to be assayed was diluted 1 in 4 prior to use and 40μl of each added in duplicate to the microtitre plate. A negative control consisting of 40μl distilled water was included. Volumes (120μl) of 12.5% aqueous Na_2CO_3 were added to all the samples including the standards followed by 20μl 0.1% $CuSO_4.5H_2O$. The

plate was left for 1h at room temperature. A solution of Folin's reagent was then diluted 1 in 3 and 20μl added to each of the samples (including the standards), the plate was left at room temperature for a further 25 min. The plate was then read in an ELISA plate reader at 750nm.

2.21 LPS core type ELISA

2.21.1 Core-polymyxin conjugate

PCP extracted LPS cores R1, R2, R3, R4 and K12 (**section 2.18**) were prepared as $1mgml^{-1}$ solutions with pyrogen free water (pfH_2O). Each LPS solution was sonicated at 5μm for 30s to aid dispersal. Polymyxin-B sulphate was prepared as a $1mgml^{-1}$ solution with pfH_2O. Volumes (1ml) of the LPS solutions were added to an equal volume of the polymyxin-B sulphate solution. The resultant solution was sonicated as before. The polymyxin-B sulphate LPS core solution was then transferred to dialysis tubing (mwco 2000; Spectrapore). The core-polymyxin solution was dialysed overnight at 4°C with constant stirring, against two litres of pfH_2O to remove any unbound polymyxin-B sulphate.

2.21.2 Coating ELISA plates

Polymyxin B-sulphate- LPS core complexes are floccular upon recovery from the dialysis tubing. Complexes were transferred to glass bijouxs and floccular masses were dispersed by sonication at 5μm for 30s. Coating buffer (0.05M sodium carbonate buffer pH9.6) was used to dilute the complexes 1 in 50 prior to coating the plate. To coat, 100μl of each complex was added to each

well of a microtitre plate (Greiner medium binding strips, Labortechnik). The coating solution was vortexed at regular intervals to prevent large LPS-polymyxin complexes becoming coated on to the ELISA plate. Coating was done overnight at 4°C.

2.21.3 Blocking

Plates were washed four times with PBS-Tween (PBS, 2.6mM KCl, 0.13M NaCl, 0.05% Tween 20, pH7.4) and then blocked overnight at 4°C with PBS + 3% fish gelatin. Plates were then washed and dried and stored at -20°C until required.

2.22 Serum preparation

2.22.1 Healthy human sera

Sera were obtained from healthy human volunteers at The University of Edinburgh. Serum was diluted 1 in 400 in PBS dilution buffer pH7.4 (4% polyethylene glycol 6000 w/v and 0.05% v/v Tween 20). Volumes (100μl) of the test sera were added in duplicate to the microtitre plate. Dilution buffer only was applied to those wells used as negative controls.

2.22.2 Healthy bovine sera

Dr Chris Low of The Scottish Agricultural College, Edinburgh provided the bovine test sera used in this study. Sera had been collected as part of the on-going screening of cattle for commercially important bovine disease. Sera represented many animals from all over Scotland. Since bovine sera had not

been used for LPS core antibody investigations prior to this study the ELISA had to be optimised.

2.22.3 Serum titration

For the determination of the optimal serum dilution for detection of anti-core LPS antibodies, bovine serum samples were first diluted 1 in 400 to screen for samples that have a median optical density reading. Each sample was applied in duplicate to five plates each coated with the core LPS-polymyxin conjugates as described in **section 2.21.2**. Samples were screened for a median value against each of the core types. Median samples were then titrated against each of the five core types. Sera was doubly diluted in PBS dilution buffer (see **section 2.22.1**) and applied in duplicate to the microtitre plate. Sera were incubated with the antigens for 90 min at 37°C. Antibody-antigen complexes were detected with sheep anti-bovine IgG HRP antibody conjugate. Colour was developed (**Section 2.22.4**) and plates were read at 450nm

2.22.4 HRP colour developing for ELISA

One tablet of tetramethylbenzidine dihydrochloride (Sigma) was dissolved per 10ml 0.05M phosphate citrate buffer (25.7% (v/v) 0.2M dibasic sodium phosphate, 24.3% (v/v) 0.1M citric cid, 50% (v/v) distilled H_2O, pH5.0). To each 10ml volume of substrate solution 2μl hydrogen peroxide was added. Volumes (100μl) of the HRP substrate were added to each well of the

microtite plate. The reaction was stopped with the addition of 50µl 2M sulphuric acid. Plates were read at 450nm.

2.23 Total immunoglobulin content.

In order to quantify the level of immunoglobulin in each of the samples used, radial immunodiffusion (RID) was used. Bovine immunolobulin (IgG and IgA) kits (Bethyl Laboratories Inc, USA) were used to quantify the samples as directed by manufacture's instructions.

2.24 ELISA for the detection of antibodies to recombinant proteins

2.24.1 Determination of optimal recombinant protein coating concentration

The availability of goat anti-GST antibody (Amersham) allowed the optimal level of GST coating to be determined. Coating was achieved by the dilution of the sample protein in 0.05m sodium carbonate buffer pH9.6 to the required concentration. Volumes (100µl) of GST at various concentrations were used to coat the microtitre plate overnight at 4°C. Negative controls received coating buffer only. Plates were blocked and washed as described previously (**section 2.21.3**). Goat anti-GST IgG (Amersham) was added at a dilution of 1 in 1,000 in 0.05M sodium phosphate buffer pH7.4, 0.85% w/v sodium chloride, 0.05% v/v Tween 20 (antiserum conjugate diluent) and plates were incubated for 90 min at 37°C. After washing, antibody antigen complexes were detected by the addition of rabbit anti-goat IgG HRP (Sigma) at 1 in 20,000 dilution in antiserum conjugate diluent for 90 min at 37°C. Plates were

then developed (**section 2.22.4**) and colour read at 450nm. Optimal coating was determined as the lowest concentration of GST that achieved maximum detection (i.e. max. OD).

2.24.2 Serum titration

Serum titration was carried out to determine the optimal dilution for detection of antibodies to Int280 and EspB. The optimal dilution is defined as the dilution that produces an optical density of half the maximum obtained. At this dilution the ELISA is most sensitive to changes in antibody titre. Microtitre plates were coated overnight at 4°C with the optimal concentration of GST and either Int280-GST or EspB-GST as described previously (**section 2.24**). Sera identified by immunoblot as having a median level of antibody reactive to Int280 and EspB were used to determine the optimal dilution. Sera were used neat, at a dilution of 1 in 100 and doubly thereafter to a final dilution of 1 in 6400 in 0.05M sodium phosphate buffer pH7.4 (Antibody buffer). Plates were blocked and washed as described previously (**section 2.21.3**). Sera at the appropriate dilution were incubated with the antigen for 90 min at 37°C. Antibody antigen complexes were detected by the addition of sheep anti-bovine IgG HRP at a dilution of 1 in 20,000 in antibody buffer. Colour was developed as described in **section 2.22.4**. Plates were read at 450nm.

2.24.3 Conjugate titrations

All Serotec antibody conjugates were titrated to determine the optimal dilution for use. Microtite plates were coated with GST, Int280-GST and EspB-GST at the optimal dilution. The same sera used in the serum titration assay (**section 2.24.1**) were prepared as previously described and used at the optimal dilution. The antibody was incubated with the antigen for 90 min. Antibody conjugates were serially diluted and incubated with the plate for 90 min. Plates were developed and read as described in **section 2.22.4.** The optimal dilution was determined as the lowest dilution that gave an OD reading equivalent to the maximum obtained.

2.25 Experimental infection with *Escherichia coli* O157

Calf challenges were performed at Moredun Research Institute (MRI) in either containment level 2 (verotoxin negative strains) or 3 (verotoxin positive strains) large animal housing facilities by staff at MRI. Calves were conventionally reared until approximately two weeks post-weaning and transported to MRI where they were acclimatized for approximately three days prior to challenge. Faecal samples were taken at least twice from each calf and confirmed negative for *E. coli* O157 by immuno-magnetic separation (IMS) prior to challenge. *E. coli* O157 strains, selected for resistance to nalidixic acid, were grown overnight in LB broth 37°C, with shaking) and diluted in sterile PBS to achieve an inoculum of 10^9 cfu per animal in a total volume of 10 ml. The inoculum was administered to the calves via a stomach tube and washed down with 500 ml sterile PBS. **Table 2.1** details

the strains used in this experiment. Three strains of *E. coli* O157 were used to experimentally infect 22 male calves (**Table 2.5**)

Table 2.5: Experimental infections

Calf number		Inoculation strain
c81	c303	Zap 198 (Walla 3),
c106	c310	Verotoxin Negative
c146	c312	
	c315	
	c323	
c87	c295	Zap 196 (Walla 1),
c95	c299	Verotoxin Positive
c111	c307	
c117	c313	
	c324	
c108		Zap 3, Lothian Red
c118		House Dairy outbreak
c121		strain (cattle isolate)
c140		Verotoxin positive

Calf numbers refer to ear-tag numbers at MRI

2.26 Sample preparation

2.26.1 Bovine serum from experimentally infected cattle

Whole bovine blood from experimentally infected cattle was collected at weekly intervals. Sera were removed and stored as previously described (**section 2.22.2**).

2.26.2 Bovine faecal samples from experimentally infected cattle

Faecal samples for the detection of IgA were treated as follows using a method adapted from Gaspari *et al* (1988). All procedures were carried out in category 3 containment facilities. Two parts protease inhibitor solution (soybean trypsin inhibitor $1mgml^{-1}$ in PBS and 50mM EDTA with 0.05%

Tween 20), were added to approximately one part (3g) bovine faecal material. 0.1M phenylmethylsulphonyl fluoride (PMSF; Sigma) in ethanol was added to a final concentration of 1mM. The mixture was vortexed for 30s and then centrifuged at 4,000g for 10 min to remove faecal debris. To the supernate, 0.1M PMSF in ethanol was added to a final concentration of 2mM. The supernate was vortexed again and left to stand on ice for 15 min. Heat inactivated (54°C for 45 min) foetal calf serum was added to a final concentration of 4% (v/v). The sample was transferred to screw-capped eppendorf tubes and centrifuged at 16,000g for 5 min. The supernate was carefully removed. Each sample was then filter sterilised by passage through a 0.2μm filter membrane (Millipore). Samples were stored at -70°C until required.

2.27 Detection of bovine antibody to recombinant antigens

2.27.1 ELISA

Microtitre strips (Greiner) were coated overnight at 4°C with recombinant antigen diluted in coating buffer (Appendix 1). Plates were blocked as previously described (**section 2.19.3**) Bovine test sera from experimentally challenged cattle were diluted in antiserum conjugate diluent. Each sample was applied in duplicate to the microtitre plates at the required concentration. After incubation for 90 min at 37°C the plates were washed 4 times and the appropriate antibody conjugate was applied at a dilution of 1 in 20,000. All antibody conjugates were purchased from Serotec and were raised in sheep against each of the bovine immunoglobulin types.

2.27.2 Dot blot for the detection of IgA in faeces

Nitrocellulose membranes were cut into rectangles of dimensions 100 x 70mm and 2μl of recombinant protein applied with a micropipette. Each membrane was blocked with 3% fish gelatin + PBS for 45 min at room temperature. The membrane was then clamped into a 96 well manifold and 100μl of each faecal suspension diluted 1 in 4 in antiserum conjugate diluent, applied to the membrane. The manifold was sealed to prevent evaporation and left overnight. A vacuum was applied to the manifold to remove the faecal suspension and the membrane was removed and washed twice in TTBS for 10 min. Sheep anti-bovine IgA HRP or sheep anti-bovine IgG1 HRP at a dilution of 1 in 1000 was applied to the membrane for 90 min at room temperature. Colour was developed using HRP colour developer.

2.27.3 Immunoblot for the detection of antibodies to recombinant proteins.

Recombinant proteins were resolved by 10% SDS PAGE. Proteins were then transferred to nitrocellulose overnight. Nitrocellulose was removed and cut into strips of dimensions 3 x 90mm. Each strip was use to test bovine serum samples for the detection of antibodies to GST, EspB and Int280. Nitrocellulose was first blocked for 45 min in 3% fish gelatin + PBS. Serum was diluted 1 in 50 in 1% fish gelatin in PBS and incubated with each strip for 90min. Negative controls received foetal bovine serum diluted as per the samples and positive controls received goat anti-GST at a dilution of 1 in

1000. Nitrocellulose was washed twice in TTBS for 10 min. Sheep anti-bovine IgG HRP was applied for 90 min at a dilution of 1 in 1000. Positive controls received rabbit anti-goat IgG HRP at the same dilution. Colour was developed using HRP colour developer.

2.28 Protocol to assess the function of bovine antibody in preventing AE lesions

HeLa cells were cultured at 37°C with 5% CO_2 in 5ml tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5%(v/v) foetal calf serum and non-essential amino acids (Sigma) used in accordance with the manufacturer's instructions. Confluent HeLa cell monolayers were harvested by trypsin-EDTA (Sigma) digestion and centrifugation at 1,500g for 10 min. Pellets were washed in 10 ml PBS and harvested by centrifugation at 1,500 g for 10 min. Cells were resuspended in 15ml DMEM and 1ml was placed into each well of a four well chamber slide (Nunc). HeLa cells were cultured overnight at 37°C with 5% CO_2 to allow confluent monolayers to form.

Overnight 5ml cultures of toxin negative *E. coli* O157 (Zap 193: See **Table 2.1**) were grown at 37°C with shaking in LB broth. Volumes (100μl) of the overnight culture were used to inoculate 5ml DMEM supplemented with 5% (v/v) foetal calf serum, non-essential amino acids and glutamine to a final concentration of 25mM. Where appropriate serum obtained from

experimentally infected calves (**section 2.26.1**) was pooled and added to a final concentration of 1 in 20 or 1 in 200.

The culture was incubated at 37°C with shaking until A_{600}=0.4. Volumes (8μl) of the 5ml culture were used to inoculate 4ml fresh DMEM + supplements (plus where appropriate, pooled serum from experimentally infected calves at the required dilution as before). DMEM was removed from the HeLa cell monolayers and replaced with 1ml of the diluted *E. coli* O157 culture. HeLa cell monolayers were incubated at 37°C with 5% CO_2 for a further six hours. Media was replaced with fresh DMEM after 3 hours.

2.28.1 Fluorescent actin staining (FAS)

Cell monolayers were washed twice with PBS and then fixed by the addition of 300μl 4% formalin for 20 min. Formalin was discarded and the cells were permeabilised with 300μl 0.1% Triton X-100 for 5 min. After three washes with 1 ml PBS 200μl of tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein-5-isothiocyanate (FITC) labelled phalloidin was added for 20 min. Throughout the incubation with phalloidin cells were kept in the dark. Cells were washed three times in PBS, mounted with fluorescent mounting medium (Dako) and observed under a Leica fluorescent microscope. Images were captured and processed using LeicaQFluoro software.

2.29 Statistics

Data was analysed by Student t-test and Chi-squared test.

Chapter Three.

Human and bovine faecal commensal *Escherichia coli* lipopolysaccharide core type study.

Aims.

Lipopolysaccharide is the dominant surface antigen of all Gram-negative bacteria. An anti-core LPS antibody response may afford some protection against *E. coli* associated disease. There is a proven association between R3 core LPS and EHEC, therefore increased exposure to R3 core LPS may elicit a protective immune response against this virotype. It is hypothesised that cattle are exposed to more R3 *E. coli* and consequently generate an anti-R3 antibody response that may provide protection against EHEC associated disease in comparison to humans who are not commonly exposed to R3 bacteria.

Therefore the aims of this chapter were:

1) To use PCR and monoclonal antibodies to determine the LPS core type of faecal *E. coli* isolates from healthy human volunteers and adult healthy cattle.
2) To determine the distribution of the five LPS core types within both commensal populations.
3) To examine sera from healthy human donors and healthy cattle for antibodies reactive to each of the five LPS core types.
4) To investigate any link between LPS core type exposure and anti-core LPS antibody levels.

Results

3.1 LPS core type detection by PCR and dot blot

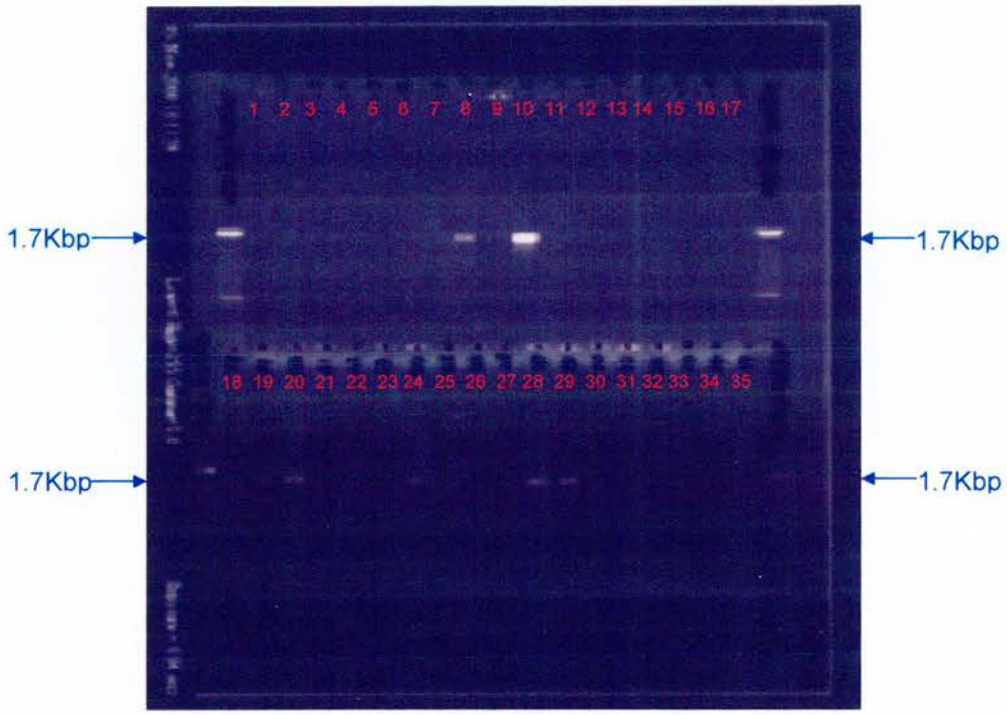
Human faecal isolates obtained from 197 healthy human volunteers were screened for LPS core type by PCR. An *E. coli* colony from each of the primary faecal cultures was selected and sub-cultured on Columbia Agar supplemented with horse blood. Bovine commensal isolates were obtained from the commensal *E. coli* collection of Dr Fiona Thomson-Carter of the University of Aberdeen (n=100) and Dr Michael Pearce of the Wellcome Trust IPRAVE study (n=100). Lysates of each of the commensal isolates were prepared and used as the DNA template for the PCR. Monoclonal antibodies were used to check the results from the PCR. **Figure 3.1a and b** show an agarose gel upon which the products of a PCR for the genes involved in R3 core LPS biosynthesis have been resolved and the corresponding dot blot developed using the W4 434.07 R3 specific monoclonal antibody (Gibb *et al*, 1992). The PCR gel (**Figure 3.1a**) shows 35 faecal *E. coli,* seven of which (lanes 8, 10, 19, 20, 24, 28 and 29) show a band at 1.7Kbp indicative of an R3 *E. coli.* The R3 specific monoclonal antibody was found to react with the same isolates and no others (**Figure 3.1b**). The controls show two positive results where R3 LPS and heat killed whole R3 *E. coli* where used. R1 and R2 core LPS was used for the negative controls; no reaction between the monoclonal antibody and these core types was observed.

Figure 3.2 shows typical results obtained from the screening of the commensal *E. coli* isolates for LPS core types R1, R2 and R4. **Figure 3.2a** shows 15 commensal isolates that have been examined for the presence of the gene coding for a specific enzyme involved in the biosynthesis of the R1 core. An R1 *E. coli* is denoted by the presence of a band at 551bp, 10 of the commensals shown are therefore R1 *E. coli*. The primers designed to detect R2 *E. coli* amplify a region of the R2 core biosynthesis producing a band at 1141bp (**Figure 3.2b**) whilst the R4 *E. coli* yield a band at 699bp (**Figure 3.2c**). The primers for the detection of R3 and K12 *E. coli* (data not shown) yield bands of 1785bp and 916bp respectively. Extra bands that appear on the gel are a result of non-specific primer binding due to the low annealing temperatures.

It was found that *E. coli* with R1 core LPS was the most frequently isolated commensal from both healthy humans and cattle (50% and 40% respectively) (**Figures 3.3 and 3.4**). The numbers of R2 and R4 *E. coli* isolated from the healthy human faeces were very similar, 12% being R2 and 14% being R4. The number of R4 *E. coli* detected among the bovine isolates was higher (20%) than in the humans. It was apparent that in humans R3 and K12 core LPS was the least common of all the core types, each being isolated in only 3% of cases. Of interest was the high level of R3 core LPS in the bovine commensals (9%) as compared to the level in humans (3%). This difference was statistically significant ($p<0.05$). Within both commensal

populations many of the organisms failed to yield any PCR products (21% humans and 19% bovines) and were consequently labelled as 'untypeable'.

a:



b:

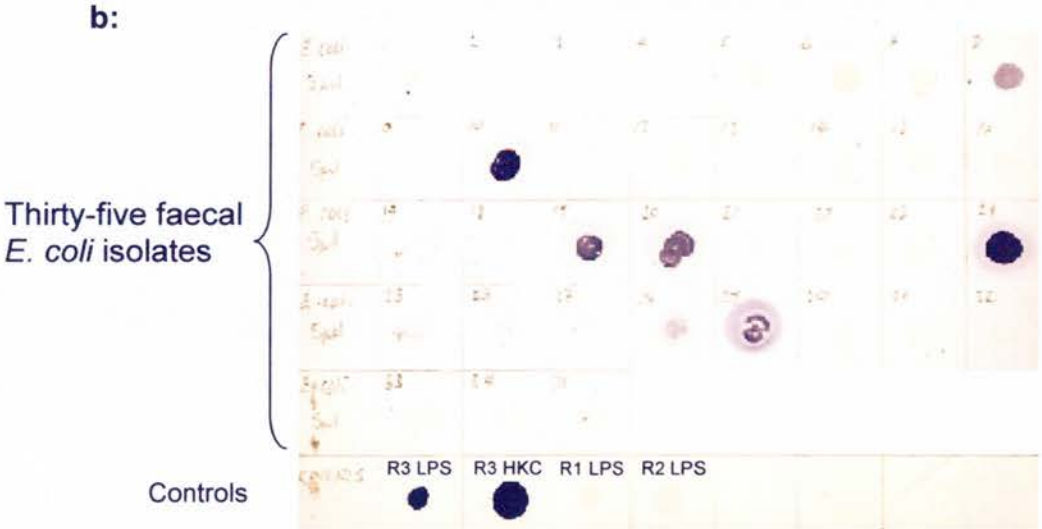


Figure 3.1a and b: Thirty-five faecal *E. coli* investigated by a) PCR and b) dot blot for R3 core LPS. **a :** Faecal *E. coli* isolates investigated by PCR for the genes involved in R3 core type biosynthesis. A band at 1.7Kbp denotes a R3 *E. coli*. Lanes 8, 10, 19, 20, 24 28 and 29 show R3 *E. coli*. **b:** The same faecal *E. coli* isolates investigated by dot blot analysis for R3 LPS. The R3 core LPS specific monoclonal, W4 434.07 was used to detect those strains with R3 core LPS. Isolates 8, 10, 19, 20, 24 28 and 29 are shown to be R3 *E. coli*. HKC – Heat Killed cells.

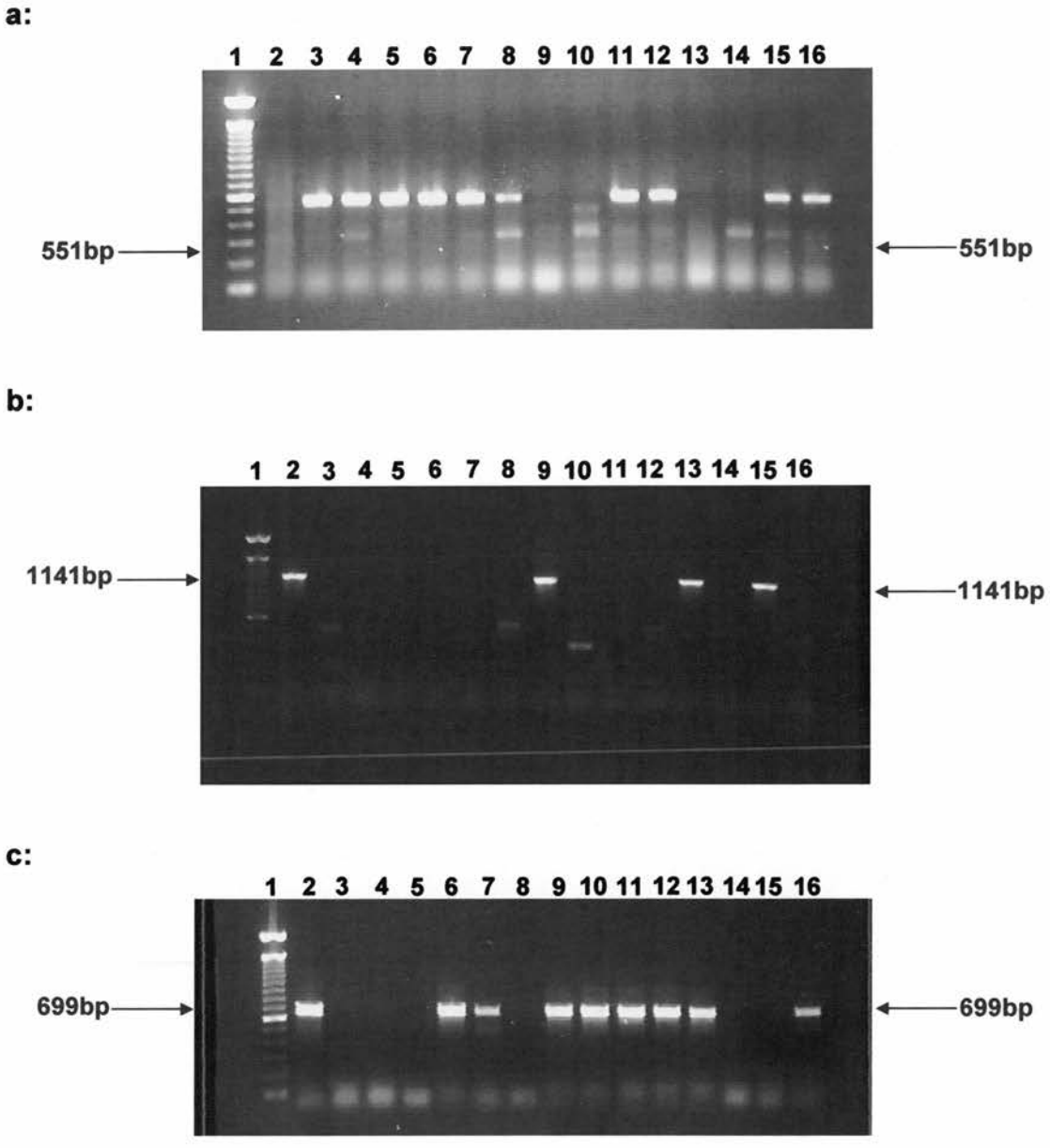


Figure 3.2: PCR for the genes involved in LPS core-type biosynthesis pathways. **a:** A band at 551bp denotes that the isolate tested carries the genes for R1 core synthesis. Ten of the samples (lanes 3-8, 11, 12, 15 and 16) are R1 *E. coli*. **b:** The genes for R2 core synthesis are detected by the presence of a band at 1141bp. Four of the samples (lanes 2, 9, 13, and 15) are R2 *E. coli*. **c:** Nine of the isolates (lanes 2, 6, 7, 9-13 and 16), show a band at 699bp. These isolates are R4 *E. coli*. In all gels the 100bp ladder is shown in lane 1.

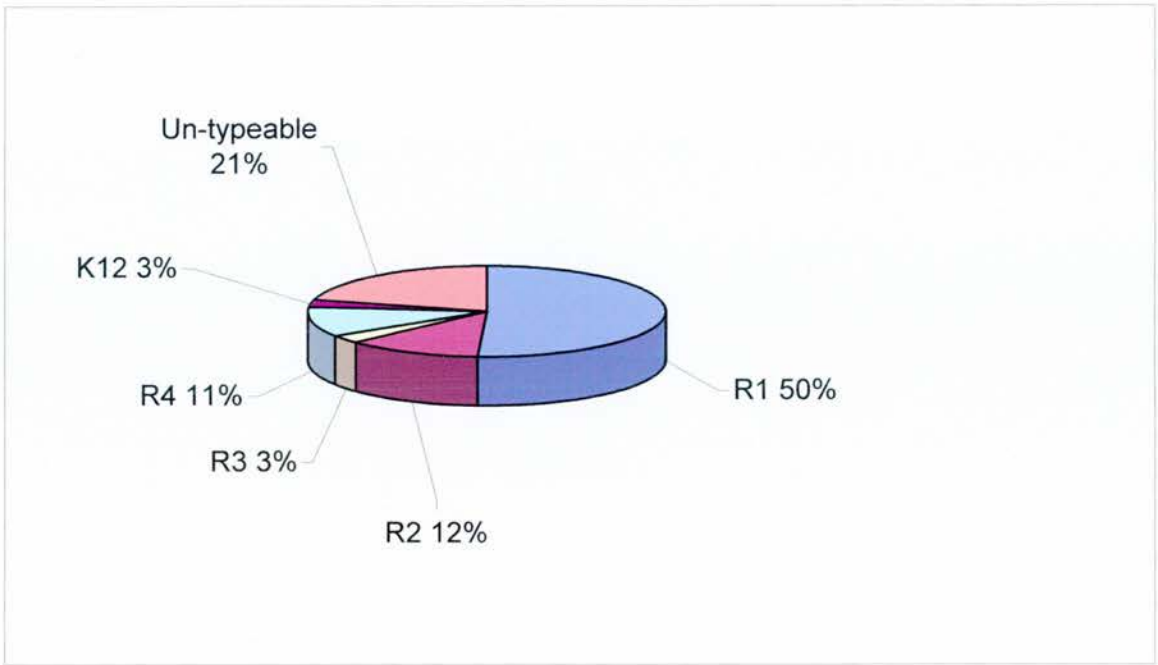


Figure 3.3: Pie chart showing the distribution of LPS core types among 197 human commensal *E. coli* isolates

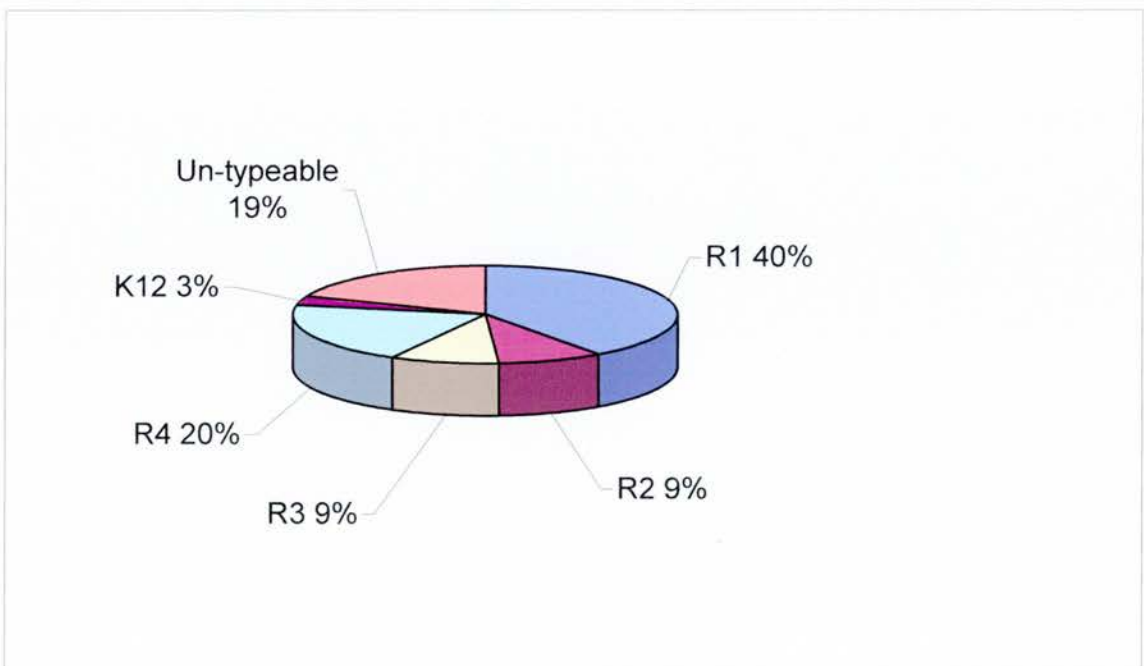


Figure 3.4: Pie chart showing the distribution of LPS core types among 200 bovine commensal *E. coli* isolates.

3.2 O-antigen variation

In order to ensure that the isolates being used in this study were not merely duplicates of the same organism, the O-antigen profiles of a selected number were investigated. Samples representing each of the core types were chosen and subjected to a rapid aqueous-phenol LPS extraction protocol based on that of Fomsgaard *et al* (1993). Each sample was then resolved by 14% PAGE and the LPS visualised by silver staining. Sixteen human isolates (**Figure 3.5**) and 19 bovine isolates (**Figure 3.6**) can be seen along with an *E.* coli O18 control for comparison. There appears to be considerable variation in the O-antigens of the isolates selected. Some of the isolates do however appear to have the same O-antigen profiles and are indeed likely to be of the same serotype, for example lanes 13-17 of **Figure 3.6** show 5 bovine isolates that probably have the same O-antigen profile. This may reflect the limited number of serotypes that exist as commensals.

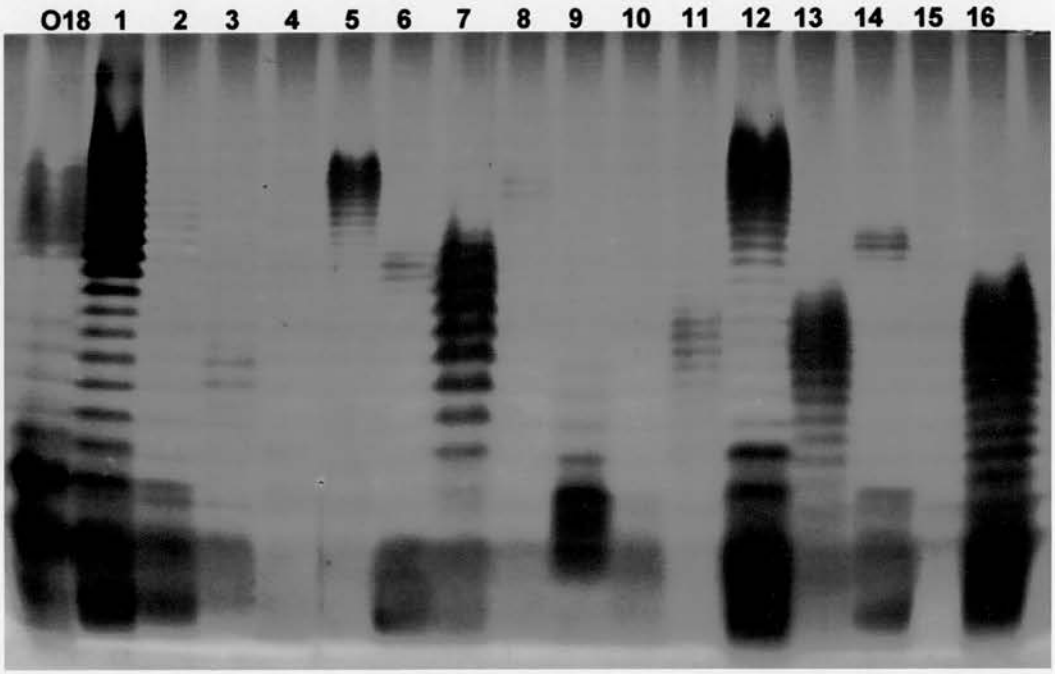


Figure 3.5: Silver stain of a 14% PAGE gel showing the LPS profiles of 16 human commensal *E. coli*

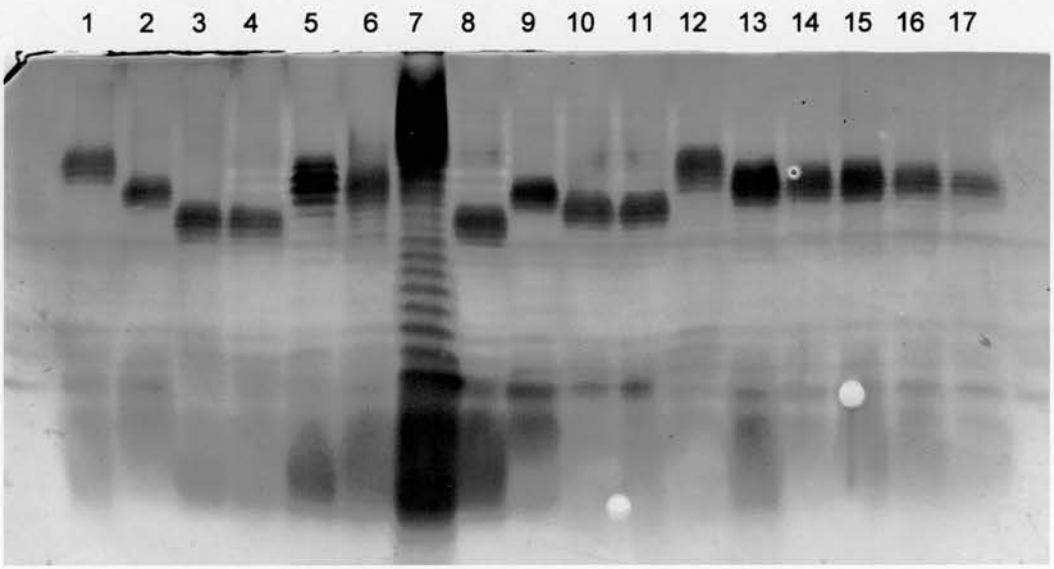


Figure 3.6: Silver stain of a 14% PAGE gel showing the LPS profiles of 17 bovine *E. coli* commensals

3.3 Bovine LPS titrations

In order to determine the optimal bovine serum dilution for the determination of LPS core type antibodies, 47 samples were screened against each of the core types. Samples were diluted 1 in 400 as per the protocol for human sera. The sample with the median optical density (OD) for each of the core types was used to create a titration curve. **Figure 3.7** shows the range of responses to each of the core types among these 47 healthy bovine samples. The black line in each of the box plots represents the median value (actual value is given beside the plot) and the green line the mean. The highest median values were obtained with the R4 and R3 core LPS whilst the R2 and K12 OD ranges were only slightly lower. All were markedly higher than the range of optical densities against R1.

The sample from which the median value was obtained was then titrated further to obtain an optimal serum dilution (**Figure 3.8**). This would be noted as the dilution that produced an OD reading equivalent to half the maximum reading obtained. Each bovine serum sample was doubly diluted and titrated against the LPS core type for which it was found to have a median OD reading. As each serum was diluted, the level of response to each core type was rapidly lost. A dilution of 1 in 4800 was enough to reduce the OD reading to background levels. Half the maximum OD reading was achieved at a dilution of approximately 1 in 1000 in each of the sera. This was determined as the optimal dilution.

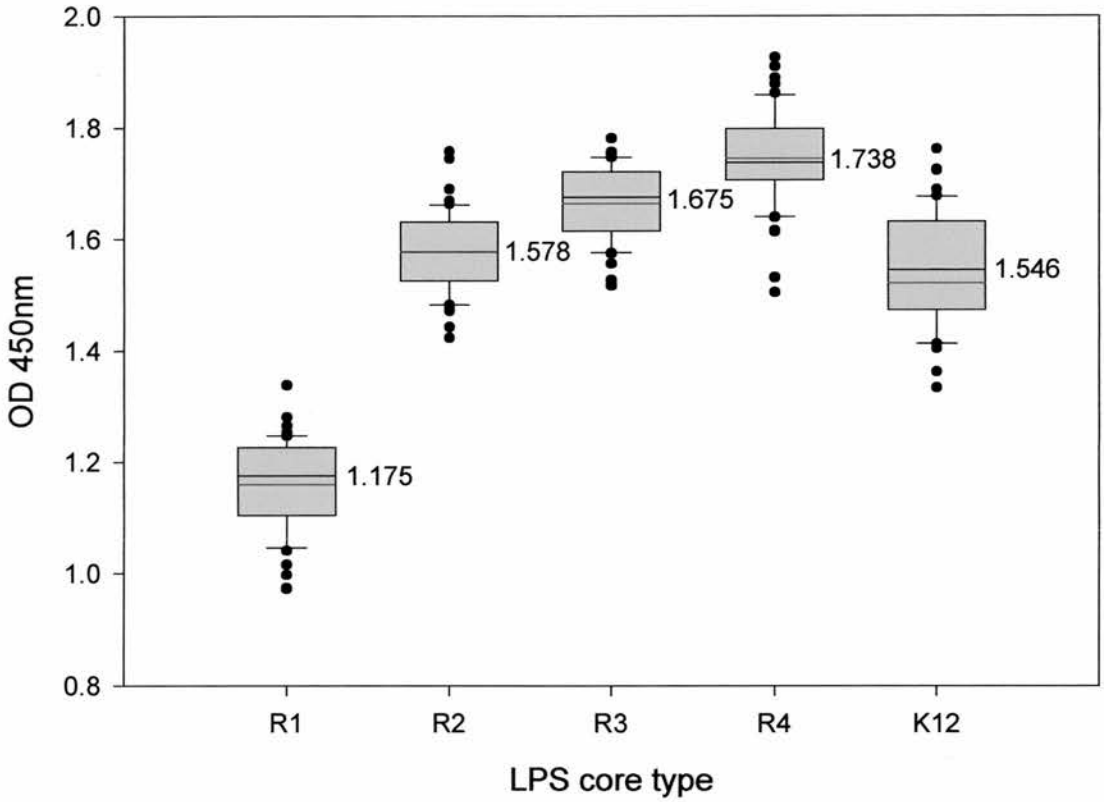


Figure 3.7 Range of antibody responses to the five *E. coli* LPS core types in 47 bovine serum samples. The black line in each of the box plots represents the median value (actual value is given beside the plot) and the green line the mean. Box plot for R2 shows only the mean value, as both the mean and median are identical. The lower boundary of the box represents the 25th percentile and the upper boundary the 75th percentile. The lines above and below the box represent the 90th and 10th percentiles respectively. Values falling out with these ranges are marked as black dots.

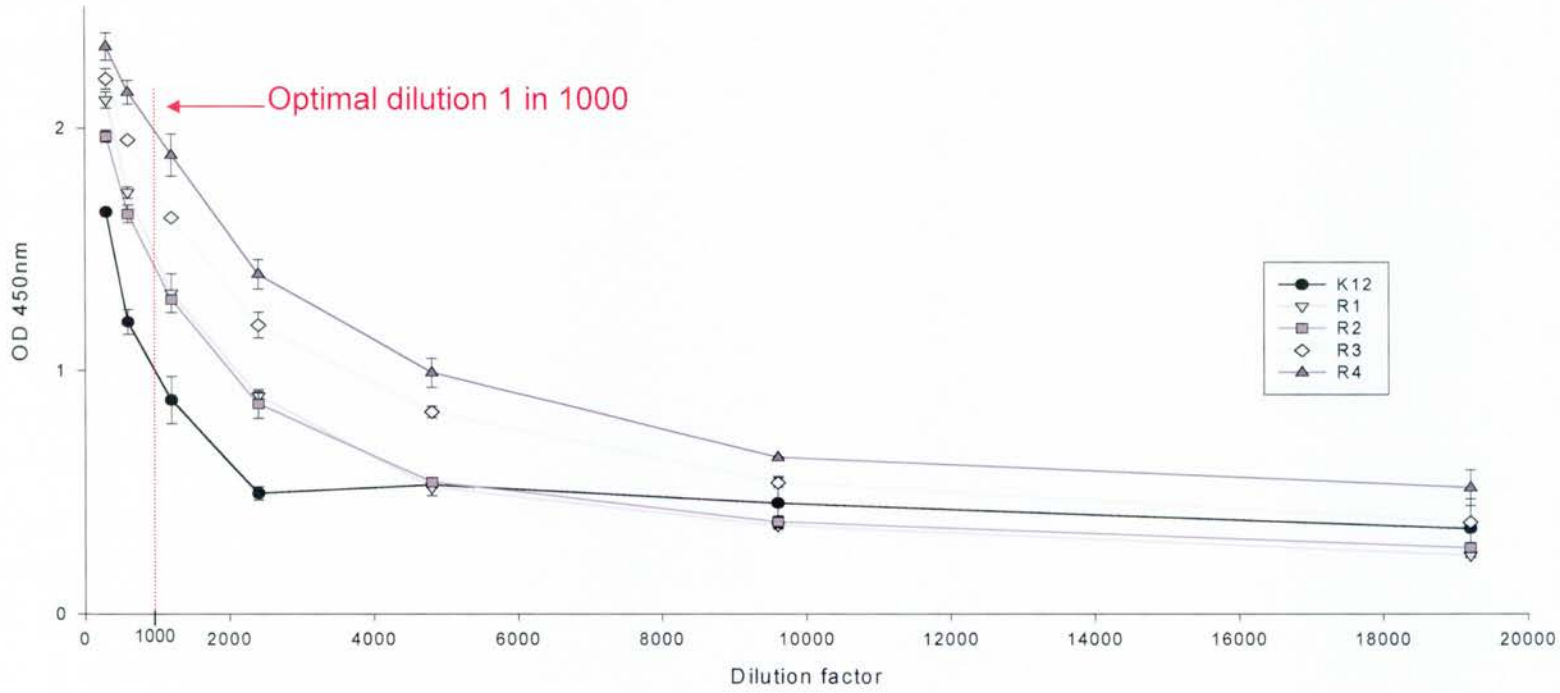


Figure 3.8: Bovine serum titration curve against each *E. coli* core type. Bovine sera exhibiting median anti-core LPS antibody titres were serially diluted to determine the optimal dilution to maximise antibody detection.

3.4 Core LPS antibody responses in healthy humans and cattle

Serum from healthy humans and cattle was used to test for the presence of antibodies reactive to each of the five known LPS core types present in *E. coli*, R1, R2, R3, R4 and K12. Core LPS-polymyxin complexes were coated on to microtitre plates. Human serum diluted 1 in 400 and bovine serum diluted 1 in 1000 was applied to microtitre plates. Antibody antigen complexes were detected with the use of either mouse anti-human IgG HRP (Sigma) or sheep anti-bovine IgG HRP. In all 91 human (**Figure 3.9**) and bovine serum samples were screened, however owing to problems incurred with the bovine standards on a number of the plates, the results obtained from only 39 of the samples tested are presented here (**Figure 3.10**). It was found that in both cases the level of anti-R4 core antibody was statistically higher than the level of any other anti-core antibody ($p<0.01$). In humans there was no statistical difference between the level of anti-R1, R2 and R3 core antibody ($p=0.3$ and 0.9). In both humans and cattle the level of anti-K12 antibody was significantly lower than any other core type response ($p<0.01$). Of particular note was the elevated level of anti-R3 core antibody relative to the R1, R2 and K12 levels among the bovine sera tested. This was found to be statistically higher ($p<0.01$) than the level of anti-R1 and R2 core antibody within the same population. **Tables 3.1 and 3.2** show all the P values obtained.

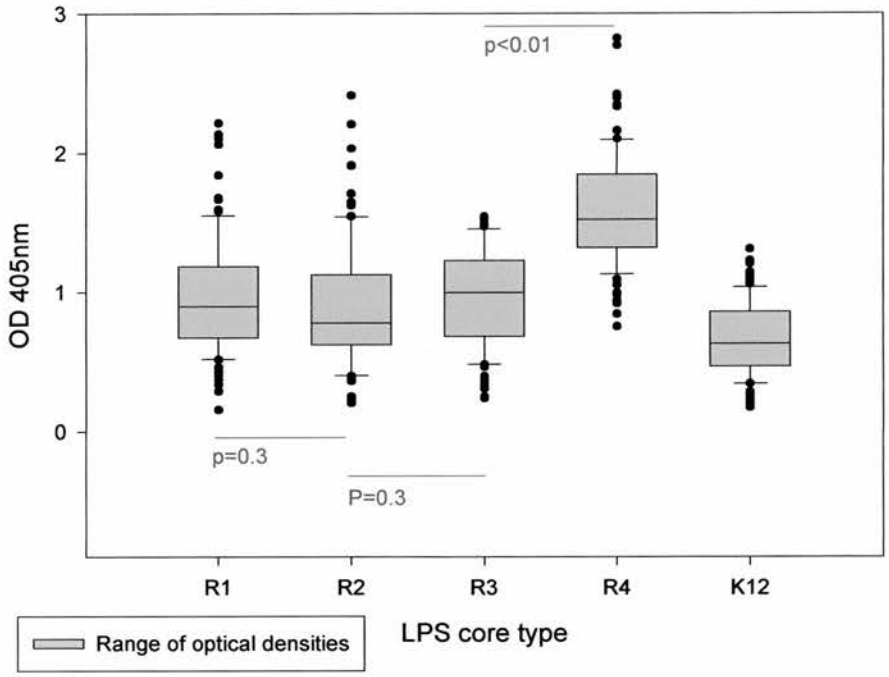


Figure 3.9: Graph to show the range of antibody responses to each of the five *E. coli* LPS core types. Tested by ELISA on 91 healthy human serum samples. Box plot parameters as **Figure 3.7**

Table 3.1: P values, obtained by student t-test, showing the significance of the antibody responses to the five *E. coli* LPS core types in 91 healthy human serum samples.

	R2	R3	R4	K12
R1	0.3	0.9	<0.01	<0.01
R2		0.3	<0.01	<0.01
R3			<0.01	<0.01
R4				<0.01

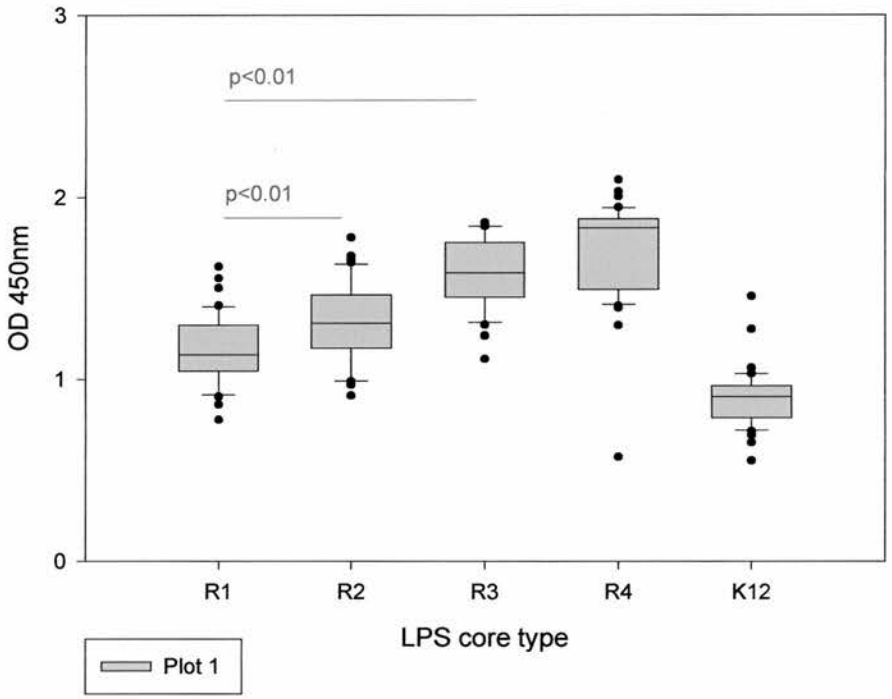


Figure 3.10: Graph to show the range of antibody responses to each of the five *E. coli* LPS core types. Tested by ELISA on 39 healthy bovine serum samples. Box plot parameters as **Figure 3.7**

Table 3.2: P values, obtained by student t-test, showing the significance of the antibody responses to the five *E. coli* LPS core types in 39 healthy bovine serum samples

	R2	R3	R4	K12
R1	<0.01	<0.01	<0.01	<0.01
R2		<0.01	<0.01	<0.01
R3			0.03	<0.01
R4				<0.01

3.5 Discussion

A previous study by Currie and Poxton (1999) showed that *E. coli* O157 and most other non-O157 verotoxin producing *E. coli* carry the LPS core type R3. The study looked at 28 verotoxigenic *E. coli* (VTEC) strains, of which 20 were O157, five were O111 with one each from serotypes O26, O128 and O86. All were found to carry R3 core LPS. More recently studies by Amor *et al* (2000) have confirmed that R3 core LPS is indeed often associated with VTEC. They reported that 81% of the VTEC isolates tested, were positive for R3 core LPS. This represented serotypes O26, O55, O91, O103 and O157. The study also showed that not all the VTEC are associated with R3 core LPS, for example several O157 isolates were found to have R1 core LPS. So while there is not a complete association of R3 core LPS with the VTEC, it is by far the most common core type associated with the group.

Gibb *et al* (1992) used core specific monoclonals to determine which core types were the most prevalent among blood, urine and faecal isolates. Of all the isolates examined the only true indication of the core type distribution among commensal *E. coli* can be obtained from the 21 faecal isolates examined. R1 core LPS was found to be most common (11 isolates), four isolates were found to carry R2 core LPS and two R3. Four were either R4 or K12 but were assigned no core type due to the lack of any anti-K12 or R4 monoclonal antibodies. Appelmelk *et al* (1994) investigated the distribution of LPS core types among 68 isolates from positive blood cultures. R1 core LPS was most frequently detected. Nine, 12 and seven strains were found to carry

R2, R3 and R4 core LPS respectively. Only 3 isolates were identified as being K12. Amor *et al* (2000) examined 72 *E. coli* isolates from the *E. coli* reference collection (ECOR) deemed representative of the genetic diversity within the species to obtain a view of the core type distribution in an unbiased population. R1 core LPS was found to be the most common core type. R2 and R3 core LPS was found in 11.1% of the isolates with R4 and K12 being found in only 2.8% and 5.6% of the isolates respectively. However the results presented by Appelmelk *et al* (1994) and those presented by Amor *et* al (2000) (although representative of the whole *E. coli* species), may not accurately reflect the distribution of core types within commensal flora. Subtle relationships between LPS core type and virulence determinants required to allow an organism to survive in a particular niche may affect the results.

While studies such as these have focused on the core types associated with a particular group of *E. coli* for example the VTEC, the study undertaken here was intended to show how the LPS core types were distributed among a population of commensal *E. coli* from both healthy humans and cattle. The results would reveal which core type humans and cattle were most frequently exposed to in health and whether or not this had any influence on the level of the anti-core LPS antibody response.

Currie *et al* (2001) showed that patients convalescing from O157 infection had elevated levels of IgA to O157 LPS that could be attributed, in part, to

the R3 core component. This implied an immune response to core LPS that may play a role in protection.

A number of studies have examined the correlation between levels of anti-endotoxin incomplete core antibody (EndoCAb) and the development of postoperative complications. Bennett-Guerrero *et al* (1997) postulated that endotoxin was one of the factors leading to pro-inflammatory responses in patients recovering from major heart surgery. Strutz *et al*, (1999) showed that low levels of anti-core LPS antibodies reduced the possibility of survival. In this study however serum was obtained upon admission to the intensive care unit, or upon diagnosis of sepsis, in which case the low antibody levels could have been attributed to a 'moping up' effect of the antibody by the organism causing sepsis. Bennett-Guerrero *et al* (1997) investigated links between postoperative complications and the level of IgG and IgM EndoCAb levels in 301 cardiac patients prior to surgery. They found that patients with low levels of IgM EndoCAb were more likely to suffer complications.

In a comparative study between healthy volunteers from Edinburgh, UK and Dhaka, Bangladesh, Hoque *et al* (2000) showed that there were significant differences in the levels of anti-core LPS antibodies between the two populations. Of note was the higher level of antibody to the R1, R3 and R4 core LPS in the population from Dhaka as compared to those in Edinburgh. This difference in antibody level may help to explain the higher levels of inflammatory bowel disease in the developed world and suggests a role for

both mucosal and systemic anti-core LPS antibodies in protection against such conditions. Could an increase in exposure to various *E. coli* serotypes lead to an increase in the level of antibody reactive to that core?

Among the human and bovine commensals examined here it was found that R1 core LPS was the most frequently detected core type. Despite the obvious abundance of R1 core LPS among our own commensals and those of cattle, the associated antibody response was not significantly different from the R2 response, and in both cases far lower than the response to the R4 core. Of the human isolates tested, only 11% were found to carry R4 core LPS as opposed to 20% of the bovine isolates; this was significantly lower than the level of R1 LPS within both commensal populations.

This apparent contradiction in the light of other studies may be explained by the serum sensitivity of these particular serotypes. Gibb *et al* (1992) investigated the serum sensitivity of the isolates used in their study. They found that 81% of the R1 isolates were serum resistant, while 68% of other core types were found to be resistant (R4 and K12 core types not included). This was thought attributable to the capsule (K1 or K5) associated with the R1 isolates. The K1 and K5 capsules are poorly immunogenic due to the fact that they are composed of molecules mimicking those of the host (Devine & Roberts, 1994).

Devine and Roberts (1994) reported that the level of serum sensitivity of a particular isolate was most likely linked to both capsule type and O-type. A wide range of serum sensitivities was found among those isolates with K1 and K5 capsules. Interestingly serotypes O7, O18 and O75 (representing R1 and R2 core types) were found to be sensitive to killing by the alternative complement pathway but not by the classical pathway. This may suggest that exposure to the LPS core was blocked by a capsule preventing effective antibody mediated-complement activation. Porat *et al* (1992) showed that the serum sensitivity of various serotypes of *E. coli* depended upon the lengths of the O-antigen polysaccharide side chains. It is possible therefore that R4 *E. coli* are often without capsule or have O-antigens that do not provide serum resistance. This would correlate with the finding here that while the frequency of R4 carrying commensals is not significantly higher than the level of either R1 of R2 isolates, there is a significantly higher antibody response to the R4 core. This could result from the antigen challenge delivered by a serum sensitive R4 organism when destroyed by both innate and acquired components of the immune system.

Using a chi-squared test it was possible to show that the level of R3 core LPS among the bovine commensals was significantly higher ($p<0.05$) than the level found in the human commensals. This would suggest that cattle are exposed to more *E. coli* serotypes carrying R3 core LPS than humans. The association of R3 core LPS with VTEC as demonstrated by Gibb *et al* (1992), Currie & Poxton (1999) and Amor *et al* (2000) would imply that this increased

R3 exposure would be due largely to a greater number of species related to VTECs, EHECs and EPECs within the commensal populations of cattle. The level of anti-R3 core antibody detected in the bovine sera was significantly higher than the R1, R2 and K12 response. Whilst no direct comparisons between the human and bovine results are possible, this trend was not seen in the human samples tested. It is possible that cattle being exposed to more R3 core LPS, generate a more significant response to this core type than humans who are infrequently exposed.

Of concern was the possibility that the strains used in the bovine core type study were duplicates of the same organism from the same animal. Any observed increase in the level of a particular core type could therefore have been due to the presence of these duplicates. In order to confirm that the isolates were different, their LPS profiles were examined by PAGE and visualised by silver staining. The O-antigen profiles of those strains selected exhibited considerable variation and therefore suggested that the isolates were not merely duplicates of the same organism although some appeared the same.

Core LPS may be divided into inner and outer regions, the outer region displaying more variation than the inner (Amor *et al*, 2000). Barclay (1995) showed that incomplete (Rc) core LPS comprising the Lipid A portion, three 3-deoxy-D-*manno*-oct-2-ulosonic acid *(KDO)* residues, three heptose residues and a single glucose, was the minimum structure required to

produce antibodies cross reactive to a large number of core LPSs. The variation in the outer core is often limited to differences in only one or two sugar residues. For example R1 core LPS differs from R4 core LPS in a single β(1⟶3) linked Glc residue [β(1⟶4) linked Gal at the same position in the R4 core] (Amor *et al*, 2000). This similarity between core types may result in a large number of cross-reactive antibodies. Hence the differences in antibody titre observed in this study (**Figure 3.9 & 3.10**) are possibly due to these often-subtle differences between core types. In which case the high level of anti-R4 core antibody observed in both healthy humans and cattle, is due to the single sugar difference in the outer core.

The differences in antibody levels detected may result from differences in the ability of each core type to coat to the wells of the microtitre plates. If, for example, R4 core had more binding affinity with the plastic of the wells then one would expect the amount of antibody detected would be much higher as opposed to a poorly binding core type. However the addition of polymyxin should even out these discrepancies.

Chapter Four.

The cloning and production of antigens from *Escherichia coli* O157

Aims

Results presented in chapter three show cattle to be more frequently exposed to R3 core type LPS than humans. Previous studies demonstrating an association between R3 core type LPS and VTEC suggest that this increased exposure results from VTEC among the bovine commensal flora. It has also been shown that a number of bovine VTEC isolates carry the LEE pathogenicity island. It is possible that exposure to verotoxin and antigens from the LEE result in a potentially protective immune response. Recombinant protein technology was used to allow investigations into immune responses to these antigens.

Therefore the aims of this chapter were:

1) To produce and purify the carboxy-terminal 280 amino acids of the *eae* gene product, Intimin (Int280).
2) To produce and purify the *espB* gene product, *E. coli* secreted protein B (EspB).
3) To produce and purify the B-subunit of Verotoxin 2 (Vt2B) from the BP-933W phage region of the *E. coli* EDL933 genome.

Results

4.1 PCR

The *eae280, espB* and verotoxin 2 B subunit (*Vt2*B) genes were amplified by PCR from *E. coli* strain EDL933; Zap 26 (see **Table 2.1**). **Figure 4.1** shows a 1% agarose gel upon which all PCR products have been resolved. Comparison with the 100bp ladder shows *Vt2*B at 312bp, the *eae*280 gene at 844bp and the *espB* gene at 914bp.

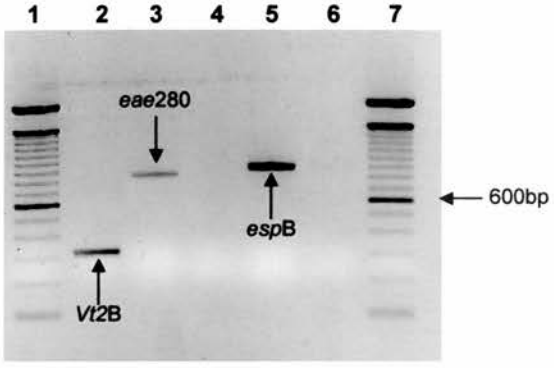


Figure 4.1: Agarose gel (1%) showing all PCR products to be cloned into pGEX-4t$_2$. The expected size of each band may be compared against the actual size as determined by the 100bp ladder (lanes 1 and 7). The sizes of each band are as follows: *Vt2B* - 312bp (lane 2); *eae*280 - 844bp (lane 3) and *espB* - 914bp (lane 5). Lanes 4 and 6 are empty.

4.2 TOPO pCR®4 cloning

After transformation of chemically competent AECC 185 *E.* coli with the ligated pCR®4 vector, colonies were examined for the presence of a cloned PCR product. Approximately 10 colonies from each plate were screened. Bacteria were grown up overnight in 5ml LBamp broth. Plasmids were extracted from the colonies by QIAprep® kits. Restriction digest with EcoR1 excised any cloned PCR product. This makes use of the two *eco*R1 restriction sites engineered either side of the cloning site on the vector, thus the excised fragment is slightly larger (18bp) than the cloned PCR product. Excised fragments were resolved by agarose gel electrophoresis. Ligation and transformation was very efficient with almost 100% of colonies examined having successfully taken up a ligated vector. **Figure 4.2** shows a typical screen of several colonies for the presence of the PCR insert. Lanes 2-6 and 12-14 show 8 colonies screened for the presence of the *eae*280 gene. The EcoR1 excised fragment appears at 862bp after agarose electrophoresis. Lanes 8-10 show three plasmids extracted from colonies transformed with the pCR®4 vector with a cloned *esp*B gene. Due to the presence of an *eco*R1 site within the *esp*B gene, this PCR product actually loses a large fragment of around 300bp. The EcoR1 excised *esp*B gene may be seen at around 600bp in lanes 8 and 10. **Figures 4.3a, b and c** detail all stages in the cloning of the primary PCR products into the TOPO pCR®4 vector. In each figure lane 1 shows the whole pCR®4 vector, the multiple bands visible are the result of plasmid DNA super coiling. Lane 2 shows the fragments generated by digestion with EcoR1. The *eae* gene (**Fig 4.3a**) can be seen at 862bp, the

*esp*B gene (**Fig 4.3b**) at 600bp with the 300bp fragment below and the *Vt2*B gene (**Fig 4.3c**) at 330bp. Lane 3 shows the BamH1 digestion profile, in this case the *bam*H1 site engineered by the primers used to generate the PCR products allow the excision of the whole PCR product. Each gel shows the BamH1 excised PCR product, the same size as those in **Figure 4.1**.

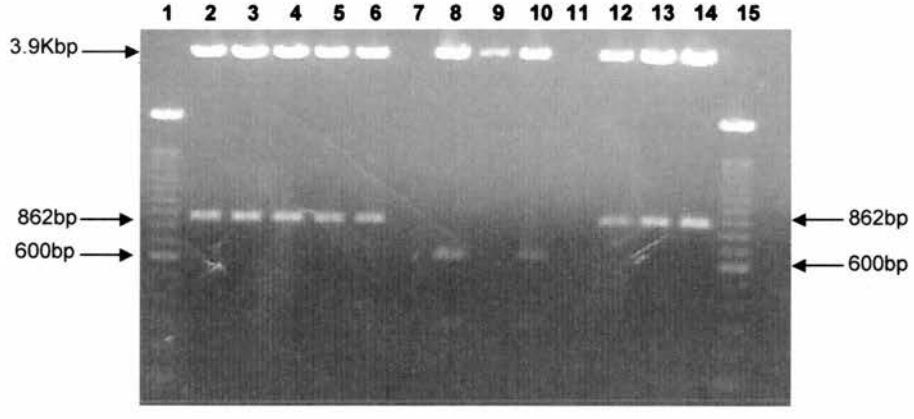
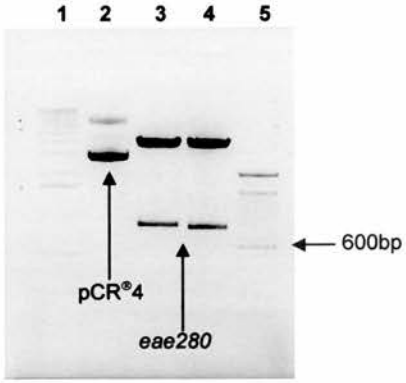


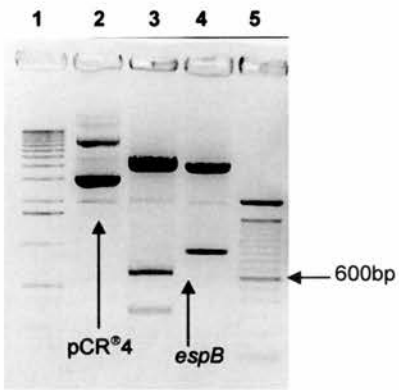
Figure 4.2: Agarose gel (1%) showing the lysate of eight colonies screened for a cloned *eae*280 gene and three colonies for the espB gene. Lanes 1 and 15 show the 100bp ladder to allow sizing. Lanes 2-6 and 12-14 are TOPO pCR®4 vectors digested with EcoR1 to excise any cloned *eae*280 genes. The excised fragment can be seen at 862bp, 18bp larger than is normal size. Lanes 8-10 show 3 plasmids digested with EcoR1 to detect any cloned *esp*B genes, 2 (lanes 8 & 10) are positive. The excised fragment may be seen at approximately 600bp, 300bp smaller than the whole fragment.

DNA markers are shown in lanes 1 and 5. The pCR®4 vector is shown in lane 2, multiple bands are due to plasmid super-coiling. Lane 3 shows the EcoR1digested plasmid (3.9Kbp) and the excised *eae*280 fragment. Lane 4 shows the BamH1 restricted plasmid and the excised *eae*A280 fragment at 844bp.

a:

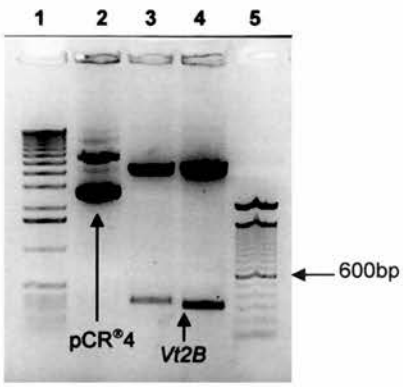


b:



DNA markers are shown in lanes 1 and 5. The pCR®4 vector is shown in lane 2, multiple bands are due to plasmid super-coiling. Lane 3 shows the EcoR1digested plasmid (3.9Kbp) and the excised *esp*B fragment at 600bp with a smaller fragment below at 300bp owing to the EcoR1 restriction site within the *esp*B gene. Lane 4 shows the BamH1 restricted plasmid and the excised *esp*B fragment at 914bp.

c:



DNA markers are shown in lanes 1 and 5. The pCR®4 vector is shown in lane 2, multiple bands are due to plasmid super-coiling. Lane 3 shows the EcoR1digested plasmid (3.9Kbp) and the excised *Vt*2B fragment at 330bp. Lane 4 shows the BamH1 restricted plasmid and the excised *Vt*2B fragment at 312bp.

Figure 4.3: Cloning into and excision from, the commercial TOPO TA cloning vector pCR®4 (Invitrogen)

4.3 Ligation

The gel-purified restriction fragments from the TOPO pCR®4 vector were resolved on a 1% agarose gel to quantify the amount of DNA present (**Figure 4.4)**. This was done by running a λHindIII marker (Invitrogen) of known DNA content (lane 1) alongside the BamH1 restricted pGEX-4t_2 vector (lane 2) seen at 4.9Kbp and the purified PCR products *espB* (914bp), eae*280* (844bp) and *Vt2B* (312bp), in lanes 3, 4 and 5 respectively. The relative intensities of the bands allow the amount of DNA to be quantified. It was estimated that the pGEX-4t_2 vector had a concentration of 10ngμl^{-1}, the *esp*B gene 5ngμl^{-1} and both the *eae*280 and *Vt2*B genes 2ngμl^{-1}.

4.4 Clone screening

After overnight incubation on LBamp plates, transformed AECC 185 colonies were screened for the presence of the vector and a cloned PCR fragment. Around 10 colonies from each plate were screened. Bacteria were grown up overnight in 5ml LBamp broth and subjected to a QIAprep® kit to extract any plasmid. The plasmid was then digested with BamH1 and the restriction products resolved by agarose gel electrophoresis. Cloned PCR products were also detected with the use of the original primers, this provided a rapid method of screening a large number of colonies. On the whole the ligation of the vector to the PCR products appeared to be very inefficient as many of the colonies were found to contain no plasmid or re-ligated pGEX-4t_2 only (a result of incomplete SAP activity). Many colonies had to be screened to obtain enough plasmids with ligated PCR products. **Figure 4.5** shows the

results of a successful ligation. Nine colonies were screened for cloned *Vt2*B PCR products. Six of the nine colonies examined here show a band at 312bp after PCR with the original primers used to amplify the verotoxin B subunit gene. Ligation, transformation and screening were repeated until at least 10 colonies containing both vector and PCR insert were identified. The orientation of the cloned PCR product in the vector was then examined.

4.5 Gene orientation

To ascertain the orientation of the cloned insert a number of restriction sites within the PCR product and the vector were utilised. Each enzyme would produce a unique fragment pattern dependent upon the orientation of the insert. **Figure 4.6** shows eight colonies that have been digested with Pst1 to determine the orientation of the *eae280* gene. This utilises the *pst*1 site within the gene itself and one in the vector. The products of seven colonies (lanes 2-5 and 7-9) show a band at 4.7Kbp and a smaller band at 1Kbp, thus the gene is incorrectly orientated. However lane 6 shows a different Pst1 restriction profile, the larger band appearing at 4Kbp and the smaller band at 1.8Kbp, this gene was found to be correctly orientated. All EspB and Vt2B clones were examined to determine the orientation of the cloned PCR product. EcoR1 was used to orientate the *Esp*B genes and Hinf1 to orientate the *Vt2*B genes. Lane 2 of **Figures 4.7a, b and c** show a correctly orientated, BamH1 excised PCR product from the pGEX-4t_2 vector. Lane 3 shows the restriction pattern obtained by restriction digest to determine the orientation of the gene in the vector. **Table 2.4** details the products expected. In **Figure**

4.7a the *eae280* gene excised from the pGEX-4t$_2$ vector can be seen in lane 2 at 844bp. Digestion of this clone with Pst1 reveals two fragments at 4Kbp and 1.8Kbp (lane 3), thus the gene is correctly orientated (see **Appendix 2** for vector maps). **Figure 4.7b** shows the EspB gene removed from the vector backbone by BamH1 digestion at 912bp (lane 2), digestion of this clone with EcoR1 revealed two fragments, one at 300bp and one at 5.4Kbp characteristic of a correctly orientated gene. Determination of the orientation of the verotoxin B subunit clones required digestion with Hinf1. Correct orientation yielded those fragments seen in lane three of **Figure 4.7c**. Correct orientation was confirmed by lack of a band at 1050bp and the presence of two bands at 1213 and 1184bp, which appear as one due to the proximal sizes.

4.6 Sequence analysis

QIAprep® kits were used to extract the plasmids from each of the correctly orientated clones. All plasmids were eluted in 50μl ultra pure H_2O and allowed to dry over night at room temperature. Plasmids were sent to MWG Biotech for sequencing. There was 100% homology between the wild-type EDL933 Vt2B subunit and Intimin nucleotide/amino acid sequence and that obtained from the fragments cloned into the pGEX-4t$_2$ vectors. Two errors were made in the EspB sequence. A guanine substitution at 50bp changed Glu_{17} to a Gly_{17}, and a thymine at 580bp changed Thr_{196} to Ser_{196}. Raw sequence data and sequence alignments may be seen in **Appendix 2.**

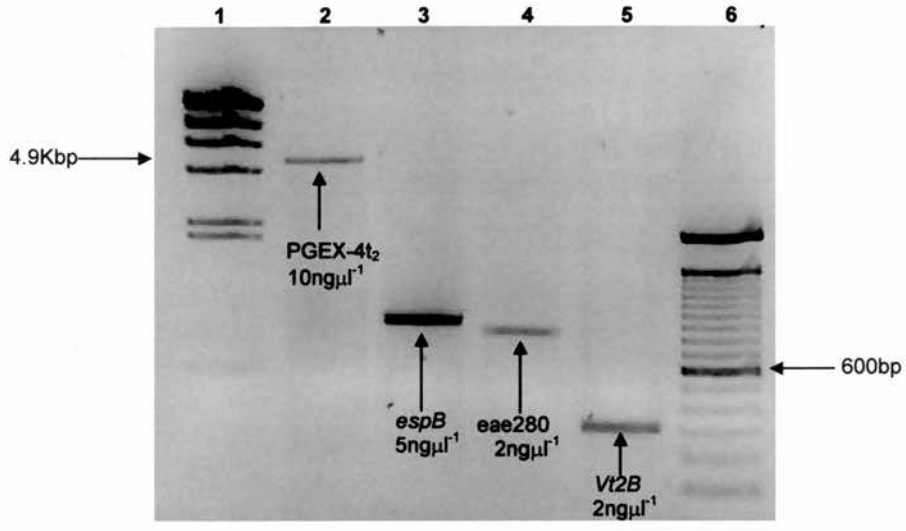


Figure 4.4. Quantification of vector and gel purified PCR product DNA by λHindIII comparison. Gel to show the quantification of DNA prior to ligation. Lane 1 shows the λHindIII marker of known DNA quantity. Each band in the gel (lanes 2-5) is then compared to the marker to obtain an approximate quantity. The values decided are as follows: pGEX4t-$_2$ (lane 2) 10ngμl^{-1}, *espB* (lane 3) 5ngμl^{-1}, *eae280* (lane 4) 2ngμlml^{-1} and *Vt2B* (lane 5) 2ngμl^{-1}. Lane 6 shows the 100bp ladder.

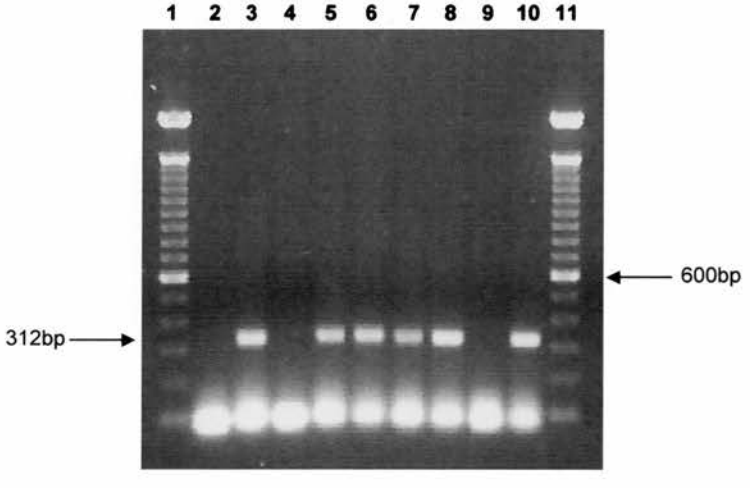


Figure 4.5: Screening for cloned *Vt2*B PCR products by PCR. Nine colonies were examined for the presence of the verotoxin B subunit genes by PCR (lanes 2-10). A band at 312bp indicates the presence of the gene. Six of the nine colonies were found to be positive. The 100bp ladder is shown in lanes 1 and 11

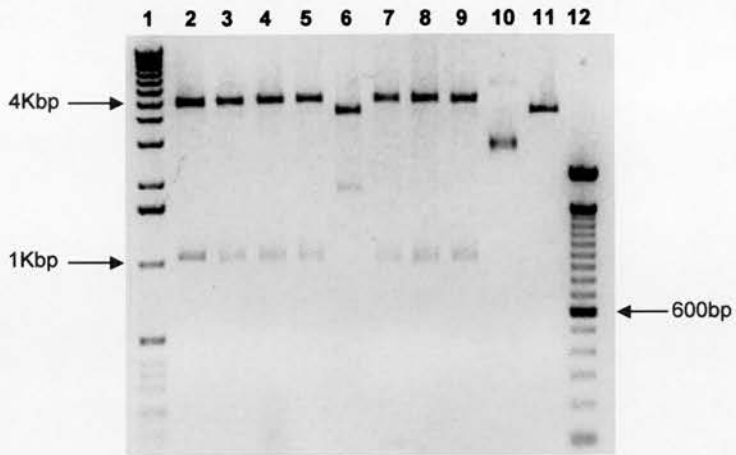
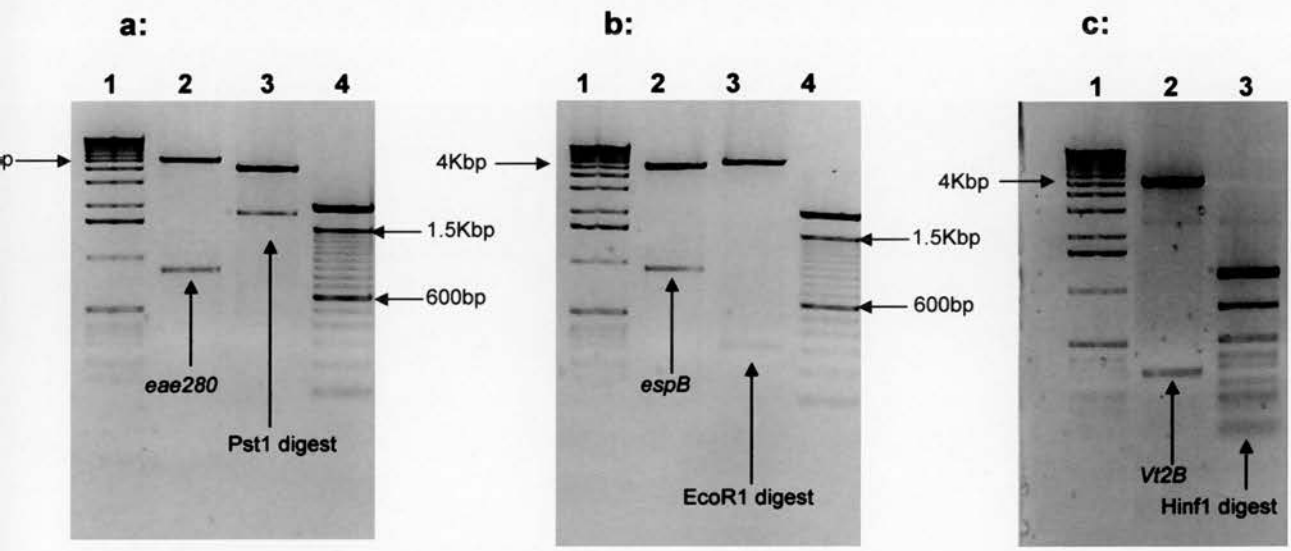


Figure 4.6: Orientation analysis of eight pGEX-4t$_2$/*eae*280 clones. Pst1 digestion of these clones determined the orientation. Lanes 2-5 and 7-9 show incorrectly orientated genes with bands at 4.7Kbp and 1Kbp. Lane 6 shows the only gene to have inserted in the correct orientation. The bands appear at 4Kbp and 1.8Kbp. A 1Kbp ladder is shown in lane 1 and a 100bp ladder in lane 12. Lanes 10 and 11 show undigested and BamH1 digested pGEX-4t$_2$ respectively.



Figures 4.7: Orientation analysis of bamH1 digested PCR products cloned into the expression vector pGEX-4t$_2$. Lane 1 in each gel shows the 1Kbp ladder. The BamH1 digested plasmid (lane 2) can be seen at just over 4Kbp in length with the excised PCR products (*eae280* at 848bp, *esp*B at 900bp and *vt*2B at 309bp) below. Lane 3 shows the restriction profile obtained by digestion with Pst1, EcoR1 or Hinf1 to determine insert orientation. Figures 4a and b show the 100bp ladder in lane 4.

4.7 Protein expression, detection and purification

4.7.1 Protein expression and detection

Overnight 5ml cultures of each clone were used to inoculate 1 litre of LBamp broth. Once cultures had reached mid-log phase protein expression was induced by the addition of IPTG to a final concentration of 1mM. Bacteria were harvested by centrifugation and subjected to sonication. After centrifugation samples of the supernate and harvested cell debris were subjected to SDS-PAGE (**Figure 4.8a**) to determine if there had been expression of GST tagged proteins. Lanes 2 and 6 of **Figure 4.8a** show the GST protein at 29KDa. Very low Vt2B expression was detected in lane 3 at around 35KDa. Much larger levels of EspB (60KDa) and Int280 (55KDA) are expressed in lanes 4 and 5 respectively. The resolved proteins were transferred to nitrocellulose and the fusion proteins detected by Western blot using a goat anti-GST IgG HRP (**Figure 4.8b**). All of the fusion proteins were located in the pellet and were likely to have formed inclusion bodies. GST expression was confirmed in lanes 1 and 5 of **Figure 4.8b** with a band at 29KDa, a faint band at 35KDa confirmed a low level of Vt2B expression, but another band was seen below at around 26KDa. High levels of EspB (lane 3 60KDa), and Int280 (lane 4 55KDa) were detected. The multiple bands below the major EspB/Int280 GST fusions are the result of host cell degradation.

All the fusion proteins and the GST alone reacted with the anti-GST antibody. The GST protein was soluble and required no further treatment. Solubilisation of inclusion bodies was achieved by urea denaturation. Fusion

proteins were re-suspended in 8M Urea + 1mM DTT and then the urea was gradually removed by dialysis as described in chapter two (**section 2.17.3**). A considerable amount of the insoluble inclusion body was released into the supernate as a result of urea denaturation and subsequent re-folding. **Figure 4.9** shows a 10% SDS PAGE upon which the original pellet sample and the urea re-natured fusion proteins have been resolved. Each insoluble fusion protein can be seen in its respective preparations in lane 2 (Int280, 55KDa), lane 5 (EspB, 60KDa) and lane 8 (Vt2B, 35KDa). No fusion protein was detected in the post sonicate supernate as seen in lanes 3, 6 and 7. By urea denaturation and subsequent re-naturation by slow dialysis most of the inclusion body was recovered from the cell debris. Lanes 4, 7 and 9 show the re-natured fusion protein after removal of precipitated protein by centrifugation at 70,000g.

4.7.2 Protein purification

Affinity chromatography columns (GSTrap FF 1ml columns, Amersham) were used to remove the GST tagged proteins. Bound fusion proteins were eluted in 20 ml 10mM reduced glutathione (sigma) plus 50mM Tris pH8. Fractions (1ml) were collected from the column and analysed by SDS-PAGE for the presence of GST tagged proteins. Volumes (5μl) of each of the fractions (1ml) eluted from the GSTrapFF column were resolved by 10% SDS-PAGE (**Figure 4.10**). The Int280 GST tagged protein can be seen in each of the first five fractions, no more Int280-GST was detected after the eighth fraction (data not shown). A number of proteins of lower molecular weight than the

major Int280/GST band (55KDa) are revealed upon Coomassie staining. Many of these are the result of fusion protein degradation within the host cell. Other proteins are contaminants non-specifically bound to, and eluted from, the column. EspB and Vt2B fusion proteins were eluted and collected the same way. SDS PAGE analysis of the fractions revealed the presence of EspB in the first 10 fractions (data not shown), however Vt2B failed either to bind, or elute from the column. No protein was detected by either SDS-PAGE or Western blot. The fractions were pooled and concentrated in 4ml Vivaspin tubes (Vivascience, Sartorius).

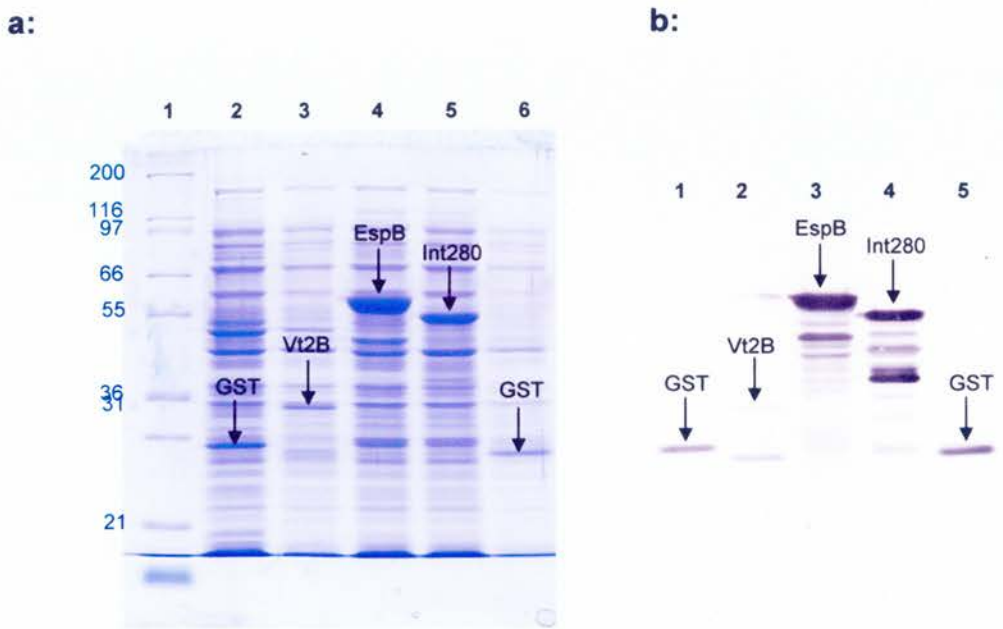


Figure 4.8: SDS PAGE and Western blot analysis of post sonicate cell debris. **a:** GST and GST fusion protein expression by IPTG induction detected in the supernate (lanes 2 and 6) and post sonicate pellet (lanes 3-5) **b:** GST fusion proteins detected by Western blot with the use of a goat anti-GST IgG HRP.

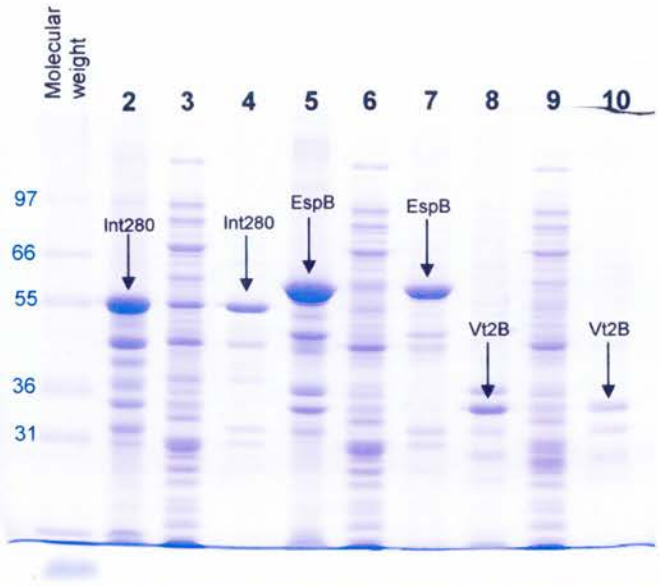


Figure 4.9: SDS-PAGE (10%) showing insoluble fusion protein before and after re-solubilisation by urea denaturation. Lanes 2, 5 and 8 show the post sonicate pellet with the large insoluble GST fusion protein inclusion body. Lanes 3, 6 and 9 show the post sonicate supernate. Lanes 4, 7 and 10 show the re-natured fusion protein now soluble and in the supernate. The molecular weight marker is shown in lane 1

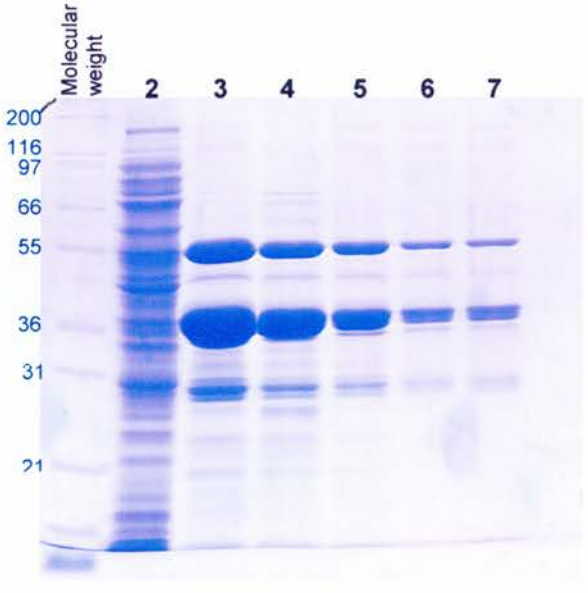


Figure 4.10: SDS PAGE (10%) showing 5μl samples of each of the first five Int280-GST fractions eluted from the GSTrapFF column. Lanes 3-7 show the protein content of progressive fractions eluted from the GSTrapFF columns. The major Int280-GST band can be seen at 55KDa, most lower molecular weight bands are the result of fusion protein degradation. Other bands are contaminants non-specifically bound to and eluted from the column. Lane 1 shows the molecular weight marker and lane 2 un-transformed *E. coli* AECC 185 (control).

4.7.3 Protein assay

Fractions eluted from the GSTrapFF columns were pooled giving a total of 20ml, which was reduced to approximately 1ml by concentration in a Vivaspin® tube (Sartorius). Prior to the assay the GST fusion protein concentrates were diluted 1 in 4 to reduce amount used. The level of protein obtained in each of the column eluates varied considerably. **Figure 4.11** shows the results obtained from the protein assay after concentration in 4ml (10,000mwco) Vivaspin® tubes (Vivascience, Sartorius). It was found that approximately 20mgml^{-1} of the GST protein had been purified. The amount of EspB and Int280 extracted was much lower at 6 and 3.5mgml^{-1} respectively

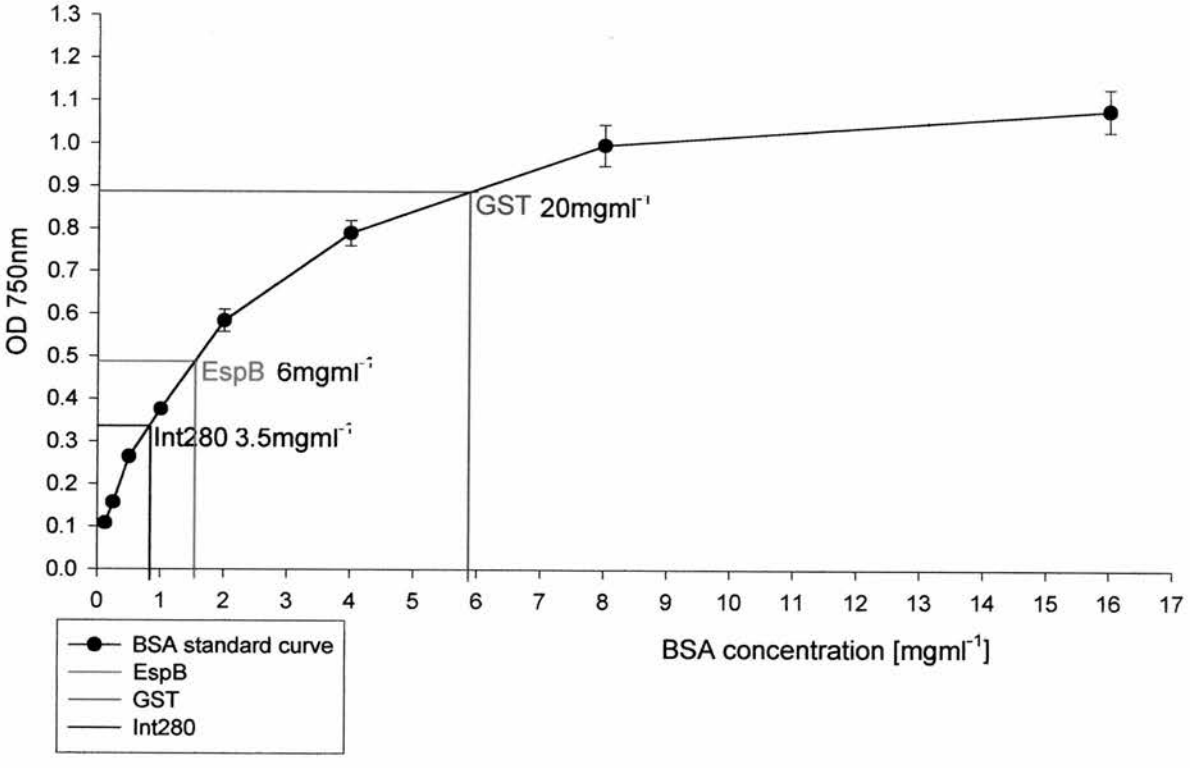


Figure 4.11: Determination of fusion protein concentration by protein assay.

4.8 Discussion

Primary PCR from the EDL933 EPEC template successfully generated products from the *eae*280, *esp*B and *Vt2*B subunit genes. Each primer had a *bam*H1 site engineered at both the 5' and 3' ends of the product to allow cloning into an expression vector. Initial attempts to digest the ends of the products with BamH1 to allow direct cloning into the pGEX-4t_2 vector were unsuccessful. This probably resulted from the restriction enzyme's (BamH1) requirement for a reasonable length of DNA upon which to sit before binding and restriction of the cleavage site (Pingoud & Jeltsch, 2001). As a result the products were cloned into the commercial cloning vector pCR®4 (Invitrogen). Almost all of the colonies examined had been successfully transformed with a ligated plasmid using this kit. Equally successful was the excision of the cloned PCR fragment from this vector using either BamH1 or EcoR1. In this case the pCR®4 vector would have provided a large amount of non-specific DNA upon which the restriction enzyme could 'sit' prior to cleavage.

The efficiency with which the pCR®4-excised PCR products were ligated to the BamH1 restricted and SAP treated pGEX-4t_2 was poor. Many colonies had to be screened before enough clones had been identified for orientation analysis. A major problem was the propensity of the restricted plasmid to re-ligate despite the use of SAP. This gave rise to many false positives on the LBamp agar plates. The β-lactamase gene encoded on the pGEX-4t_2 allowed *E. coli* transformed with the vector to secrete a β-lactamase into the agar, this prevented the ampicillin in the agar having any effect upon those colonies.

However the secretion of the β-lactamase allowed the growth of 'satellite' colonies around the transformed *E. coli*. These were often the cause of the false positives.

The orientation of the cloned PCR fragments in the vector was ascertained. Since both ends of the PCR product and the vector had been digested with BamH1, the PCR product could insert either way round. In the incorrect orientation the gene would not be transcribed properly. In more than 50% of the plasmids examined the gene cloned the wrong way. Only two correctly orientated *esp*B genes and one *eae*280 gene were identified that had cloned in the correct orientation. This trend was particularly noticeable when attempting to clone the *Vt*2B gene. A very large number of colonies had to be screened before a correctly orientated verotoxin B subunit gene was found. There appeared to be a selection pressure against the gene cloning in the desired orientation.

Previous studies by Gunzer and Karch (1993) showed that the cloning and production of this antigen was difficult. They found that cloning of the A subunit of the gene was lethal to the cell and that in order to obtain any amount of the B-subunit the 5' leader sequence had to be removed and primers were designed to amplify the region downstream of this. The result was large quantities of a truncated 4KDa verotoxin B subunit fused to GST. The loss of the 3KDa fragment would undoubtedly affect the immunogenicity of the B-subunit and therefore this approach would have been undesirable.

Acheson and Breucker (1994) showed that production of recombinant verotoxin B-subunit was possible even with the leader sequence attached. However they reported that the amount of toxin they were able to purify was well below levels achieved for other proteins. It appeared that the high level of protein expression achieved under the *tac* promoter, led to toxic amounts of the B-subunit accumulating in the host *E. coli* eventually causing lysis. The pGEX-4t_2 vector used here also contains a *tac* promoter, however cell lysis was not observed. *E. coli* transformed with a pGEX-4t_2 vector ligated to a correctly orientated *Vt2B* gene did appear to grow less well than *E. coli* transformed with other pGEX-4t_2 plasmids. In an attempt to reduce the possibility of lysis the induction temperature was reduced to 35°C slowing the growth of the *E. coli* and hence the rate of transcription. Despite this we were unable to induce large amounts of the verotoxin 2 B-subunit.

Byun *et al* (2001) utilised *Bacillus brevis* as a secretion-expression system to obtain recombinant verotoxin 1 and 2 B subunit. The promoter and signal peptide-encoding region of a cell wall protein of *B. brevis* in the pNU212 vector used, allowed secretion of the recombinant protein into the culture supernate. Despite successfully extracting Vt2B from the supernate it was found that the recombinant protein was unable to bind to its receptor globotriaosylceramide$_3$ (Gb_3). Indeed subsequent mouse immunisation experiments by Byun *et al* (2001) showed that unlike the recombinant Vt1B, the recombinant Vt2B failed to generate an immune response. It is possible

that conformational changes in the recombinant Vt2B produced by this system affected the antigenicity of the protein.

Western blot showed the Vt2B-GST as a very faint band at 35KDa. Associated with this band was another at 26KDa, which due to its strong reaction with the goat anti-GST IgG was identified as GST. **Figure 4.12** shows a Western blot in which the proteins transferred to the nitrocellulose were incubated along with an anti-GST antibody. The GST protein is shown as a band at 29KDa, while the Vt2B-GST fusion appears at 35Kda. The smaller band that appears below this reacted well with the anti-GST antibody. In its natural state GST exists as a 26KDa protein, however when expressed from a parental vector such as pGEX-4t_2 will appear as a 29KDa protein. The band at 26KDa below the Vt2B-GST protein is GST as it occurs naturally. This would imply that the 26KDa fragment was the result of a truncated read-through of the whole fusion protein or that the fusion protein was being cleaved, releasing GST. This would explain the low level of Vt2B-GST transcription seen at 35KDa, as not all the proteins transcribed are likely to be degraded. If the fusion protein was being broken down we might have expected to see a smear on the western blot relating to all the GST intermediates between the 29KDa and 26Kda GST. Instead we see a very tight band.

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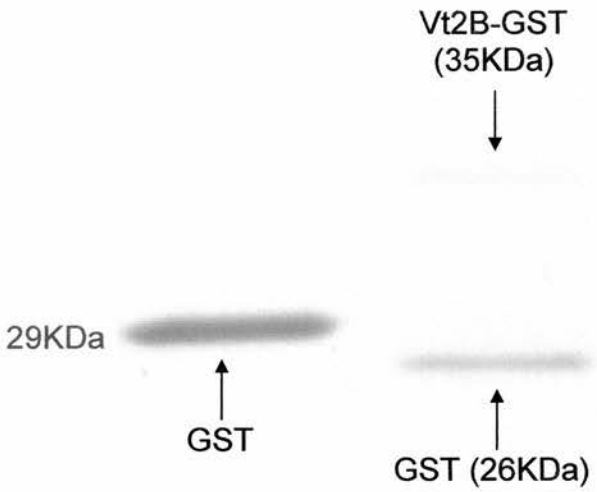


Figure 4.12: Detection of Vt2B fusion proteins after IPTG induction. Western blot showing GST at 29KDa as induced from pGEX-4t_2 by IPTG and recombinant Vt2B subunit at 35KDa with a smaller 26KDa GST fragment below.

Vt2B could not be bound or eluted from the column. Sequence analysis of the cloning region on the pGEX-4t_2 plasmid showed that the 312bp fragment ligated to the pGEX-4t_2 plasmid had 100% homology with the *Vt2B* gene from the *E.* coli strain EDL933. It is likely that the 36KDa GST protein when passed through the affinity purification (GSTrap FF) column, binds the glutathione substrate attached to the Sepharose 4B in the columns. The binding capacity of the columns is very low (~1mg) and hence it would only take a small amount of GST to occupy all the binding sites or at least out compete the Vt2B-GST fusion. If half the Vt2B were bound and the rest lost in the flow through then this would probably reduce the level of Vt2B present to below the limits of SDS-PAGE and immunoblot detection. Alternative methods of purification may have to be considered in order to obtain good yields of the B subunit fusion from this clone. One approach is to bind the Vt2B to Gb_3 conjugated to sepharose (Nakajima *et al*, 2001). This would

abolish the problems associated with the 26KDa GST fragment. Another would be to transform a VTEC strain of *E. coli* such as *E. coli* O157 with the pGEX-4t_2-Vt2B vector. It is possible that these VTEC strains may be better-suited cope with the production of Vt2B.

Despite problems with the purification of the recombinant verotoxin B-subunit, there were no such difficulties with either Int280 or EspB. Once correctly orientated clones had been identified, production and purification was relatively simple. In fact transcription of the recombinant proteins was so efficient that it resulted in the formation of inclusion bodies. In order to extract the recombinant protein it had to be denatured using urea. The recovery of the re-natured recombinant protein from the supernate was highly effective with almost 80% of the protein being recovered. While the actual conformation assumed by the recombinant proteins after re-naturation could not be determined, it was hoped that the removal of the urea by slow dialysis and the inclusion of dithiothritol (DTT) allowed correct folding. The GST appeared to bind well to the affinity purification columns suggesting that the folding of the protein was correct. The production of recombinant Int280 and EspB is well documented in the literature. Most prefer to produce recombinant proteins vectors that result in recombinant His-tagged proteins. Jenkins & Chart (2000) used pET-28 vectors (Novagen) to generate His-tagged Int280, EspB, Tir and EspA. The advantage of their system is that purification is achieved with the use of a small tag. The size of the tag (6-10 His residues) is unlikely to have as much of an effect upon the overall

conformation of the recombinant protein as the large 26KDa GST moiety. Attempts were made to clone into the pET-28a however this was unsuccessful.

Chapter Five

Antibody responses to surface and secreted components of *Escherichia coli* O157:H7 in experimentally infected calves.

Aims

Recombinant antigens generated in chapter four along with outer membrane and secreted protein preparations will be used to examine sera from experimentally infected calves for the presence of antibodies reactive to a number of antigens from *E. coli* O157.

Therefore the aims of this chapter were:

1) To detect antibody responses in the sera of experimentally infected calves to two recombinant proteins, Intimin280 (Int280) and EspB.
2) To detect antibody responses to the outer membrane proteins of *Escherichia coli* O157
3) To detect antibody responses to the secreted proteins of *Escherichia coli* O157
4) To detect responses to the LPS of *Escherichia coli* O157
5) To detect IgA responses to Int280 and EspB in faecal antibody preparations from experimentally infected calves

Results

5.1: Immunoblot analysis

5.1.1: Detection of antibody responses to GST, Int280 and EspB in experimentally infected calves

Recombinant GST, Int280-GST and EspB-GST were resolved by SDS-PAGE. Staining with zinc chloride allowed the proteins to be visualised prior to transfer to nitrocellulose. The Int280-GST (**Figure 5.1**) appears as a 50Kda band with associated smaller proteolytic fragments beneath. The recombinant EspB-GST (**Figure 5.2**) appears as five bands on the gel. The first band (approximately 53KDa) is the whole EspB-GST fusion; those below are the result of protein degradation. The GST appears as a single band at 29KDa (data not shown)

The GST, Int280 and EspB fusion proteins were transferred to nitrocellulose. The nitrocellulose was cut into strips (3mm x 90mm) and each strip used to detect antibodies to GST, Int280-GST or EspB-GST in the sera of experimentally infected calves. Sera were diluted 1 in 50 in antibody diluent prior to incubation with the nitrocellulose. Antibody-antigen complexes were detected with the use of sheep anti-IgG HRP. Each calf in the experiment was bled prior to and weekly for four weeks after an oral challenge with 10^9 *E. coli* O157. **Figure 5.3** shows the level of antibody reactive to GST in 11 experimentally infected calves. All of the calves examined had an apparent low level of antibody reactive to GST. The level of the response did not change as the experiment progressed. In some of the animals there are

bands that do not appear in the positive control and are therefore contaminating proteins that have been eluted from the column along with the GST.

Figure 5.4 shows the level of antibody reactive to the Int280-GST fusion in the same 11 animals. The positive controls (lanes 2 and 14) pick out the bands of interest. The major band at around 50KDa represents the whole Int280-GST fusion. The minor bands beneath this are a result of fusion protein degradation during production in the host cell. The negative control shows no significant bands although there are very faint reactions to some of the Int280-GST proteins. All the animals tested had antibodies that reacted to the major Int280-GST fusion. The lack of a response to GST alone would suggest that these are antibodies specific to Int280. A number of the smaller proteolytic Int280 fragments appear to be immunogenic in some of the calves. For example c108 (lane 7) and c121 (Lane 11) appear to have different anti-Int280 IgG profiles. To show that the immune response to Int280 did not vary at all during the experimental challenge, the sera of several of the animals from each week of the experiment were tested for antibodies to both GST and Int280. **Figure 5.5** shows the immune response in calf c81 to Int280 at weekly intervals during a four-week experimental challenge. The positive control shows all the Int280 fusion proteins present on the nitrocellulose. The pre-bleed anti-Int280 IgG status of c81 is shown by the blot in lane 1. There are good reactions to most of the bands shown in the positive control. A number of the smaller proteolytic Int280/GST

fragments have not reacted with the sera. After experimental infection and over the next four weeks, there was no change in the immune response. This trend was seen with the other animals examined. No response to GST alone was observed.

The EspB-GST fusion can be seen in lanes 3, 15 and 16 of **Figure 5.6** (positive controls) at just over 50KDa with a number of minor bands below. These result from the degradation of the whole EspB-GST fusion. Two negative controls were included here. Lanes 1 and 2 show the negative controls obtained with foetal calf serum and antibody diluent. None of the EspB-GST proteins can be seen to react with either the foetal calf serum or the antibody diluent. The sera from these animals was already shown to have a negligible level of antibody to GST (**Figure 5.3**), hence any band seen on the test strips that is also present on the positive control strips, must be attributable to anti-EspB IgG. The apparent level of response to EspB is markedly lower than to Int280. Many of the animals, for example c81, c111 and c117, fail to show any response to EspB at all. Calf c140 appears to have antibodies to EspB both before and after challenge. The level of response in this animal appears not to have increased over the experimental period. Calves c108 and c121 show an antibody response to a contaminating protein eluted from the column. Calf c95 (lane 6) appears to have generated an antibody response to EspB. The pre-bleed from this animal shows no visible reactivity to any of the EspB-GST proteins, but the 4-week post challenge serum shows a good response.

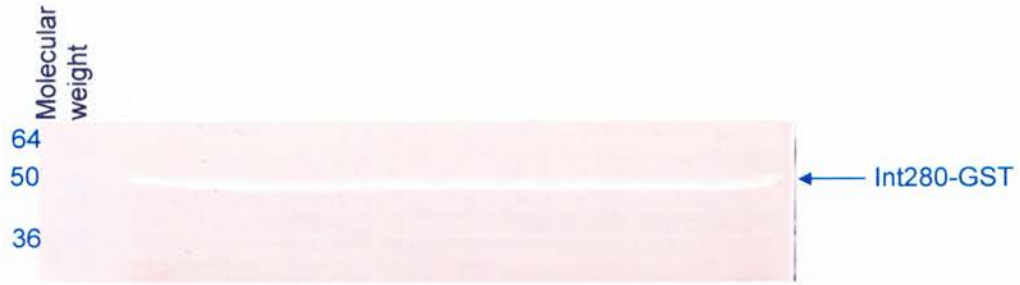


Figure 5.1: Purified Int280-GST resolved by SDS PAGE prior to transfer to nitrocellulose Purified recombinant Int280-GST resolved by 10% SDS-PAGE. The whole Int280-GST fusion protein appears as a single band at 50KDa. The faint bands beneath are a result of host cell fusion protein degradation.

Figure 5.2: Recombinant EspB resolved by 10% SDS PAGE prior to transfer to nitrocellulose. Purified recombinant EspB-GST resolved by 10% SDS-PAGE. EspB-GST appears as five bands on the Gel. The first band at just over 50Kda is the whole EspB/GST fusion protein; those beneath are the result of protein degradation.

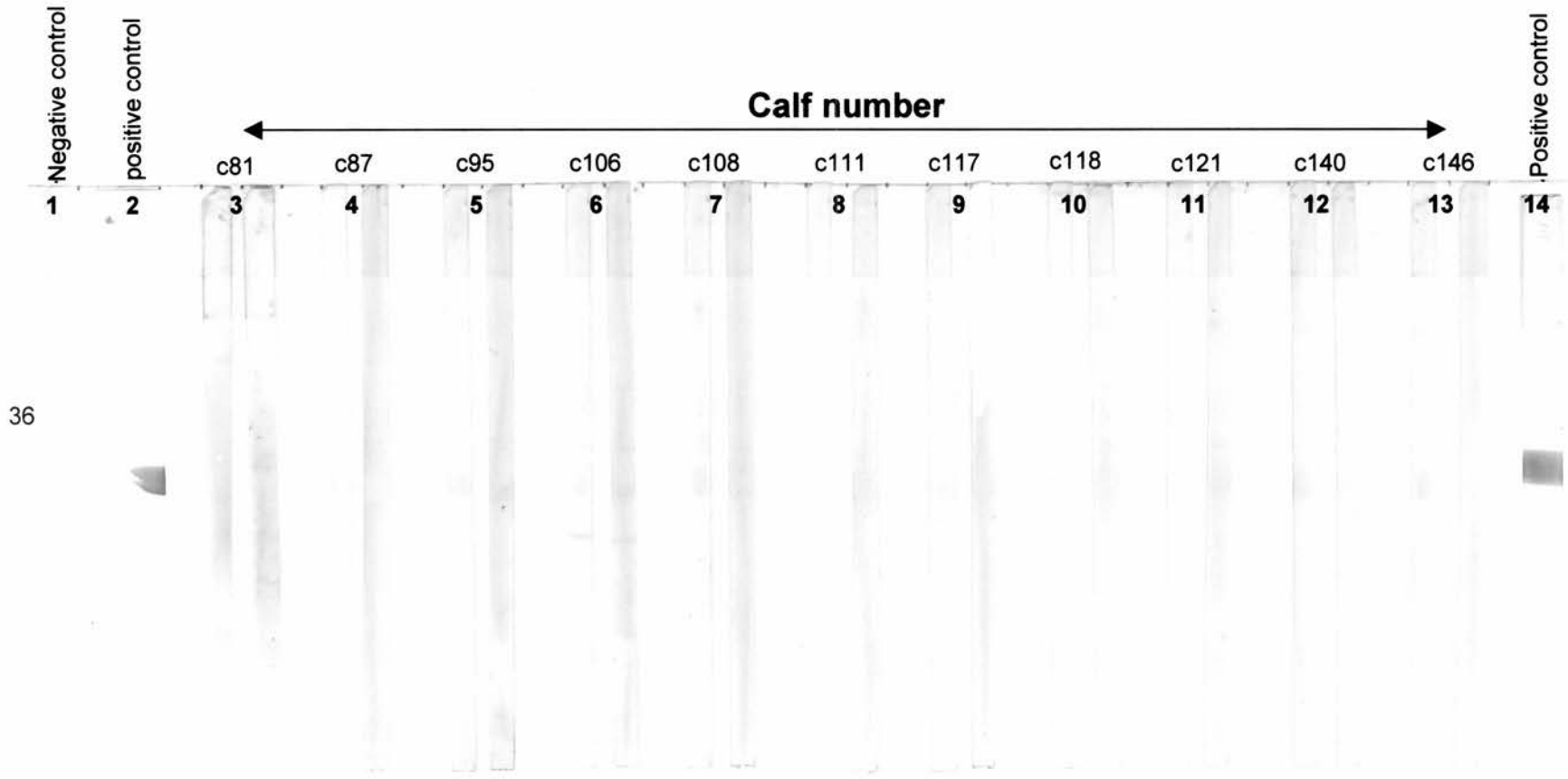


Figure 5.3: Immune response to Glutathione S-transferase (GST) in 11 experimentally infected calves. The positive controls (lanes 2, 3 and 14) were generated using a goat anti-GST IgG (Amersham). In this case the blot shows that GST forms a single band at 29KDa.The negative control (Lane 1) was generated using foetal calf serum, no bands are visible. Lanes 3-13 show the pre and 4 week post challenge anti-GST IgG status of 11 experimentally infected calves. A faint reaction to GST is visible in all the animals.

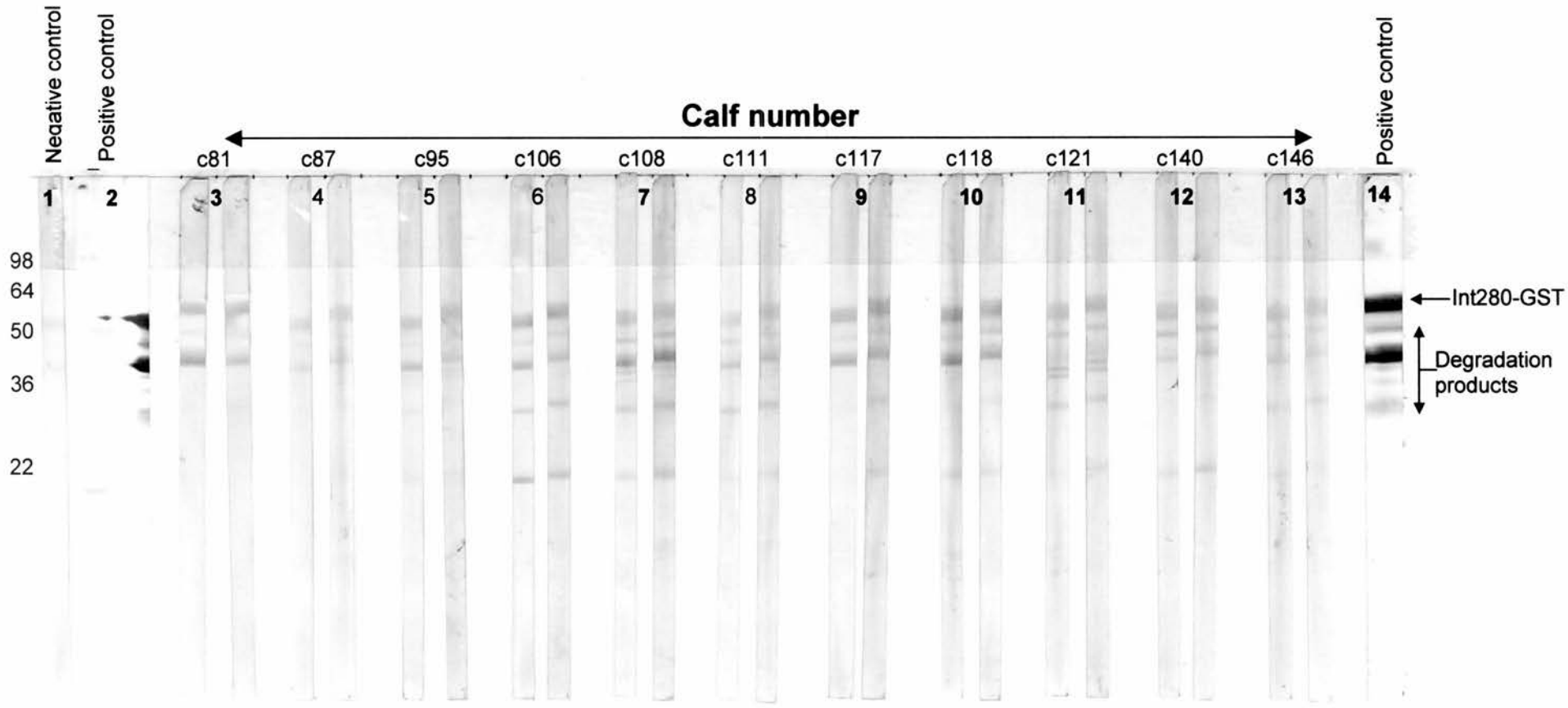


Figure 5.4: The immune response to Int280 in 11 experimentally infected calves. The positive controls in lanes 2 and 14 show the bands of interest. The major Int280/GST band can be seen at approx. 55KDa, proteolytic fragments of the whole fusion result in the smaller bands seen beneath this 55KDa protein. The negative control (lane 1) shows that while there is no significant reaction to any of the proteins there are some faint bands. Lanes 3-13 show pairs of blots that represent the pre and week-4 post challenge anti-Int280 IgG status of 11 experimentally challenged calves.

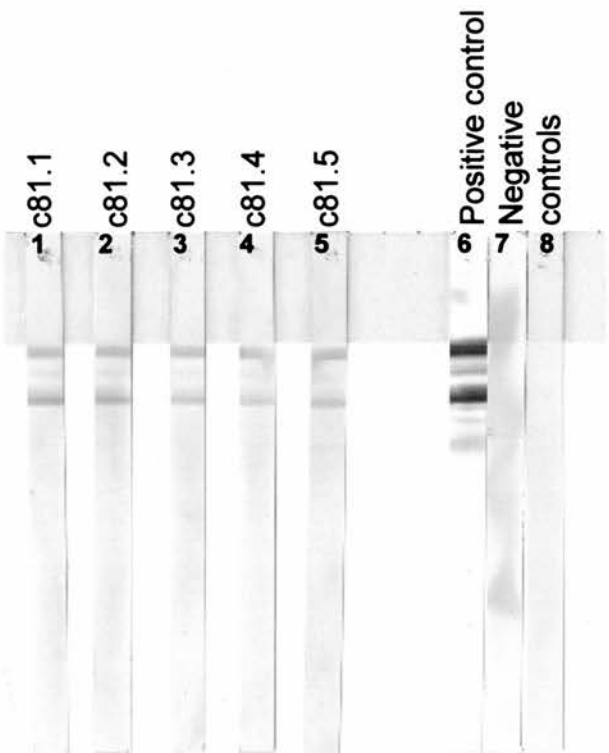


Figure 5.5: The immune response to Int280 in calf c81 at each week during an experimental infection. The series of blots in lanes 1 to 5 represent the anti-Int280 IgG status of calf c81 during a four-week experimental infection with *E. coli* O157. Lane 1 shows the animal's status prior to challenge and lanes 2-5 the status each week thereafter. The positive control (lane 6) shows all the Int280 bands. The negative controls (lanes 7 and 8) show no significant reactions.

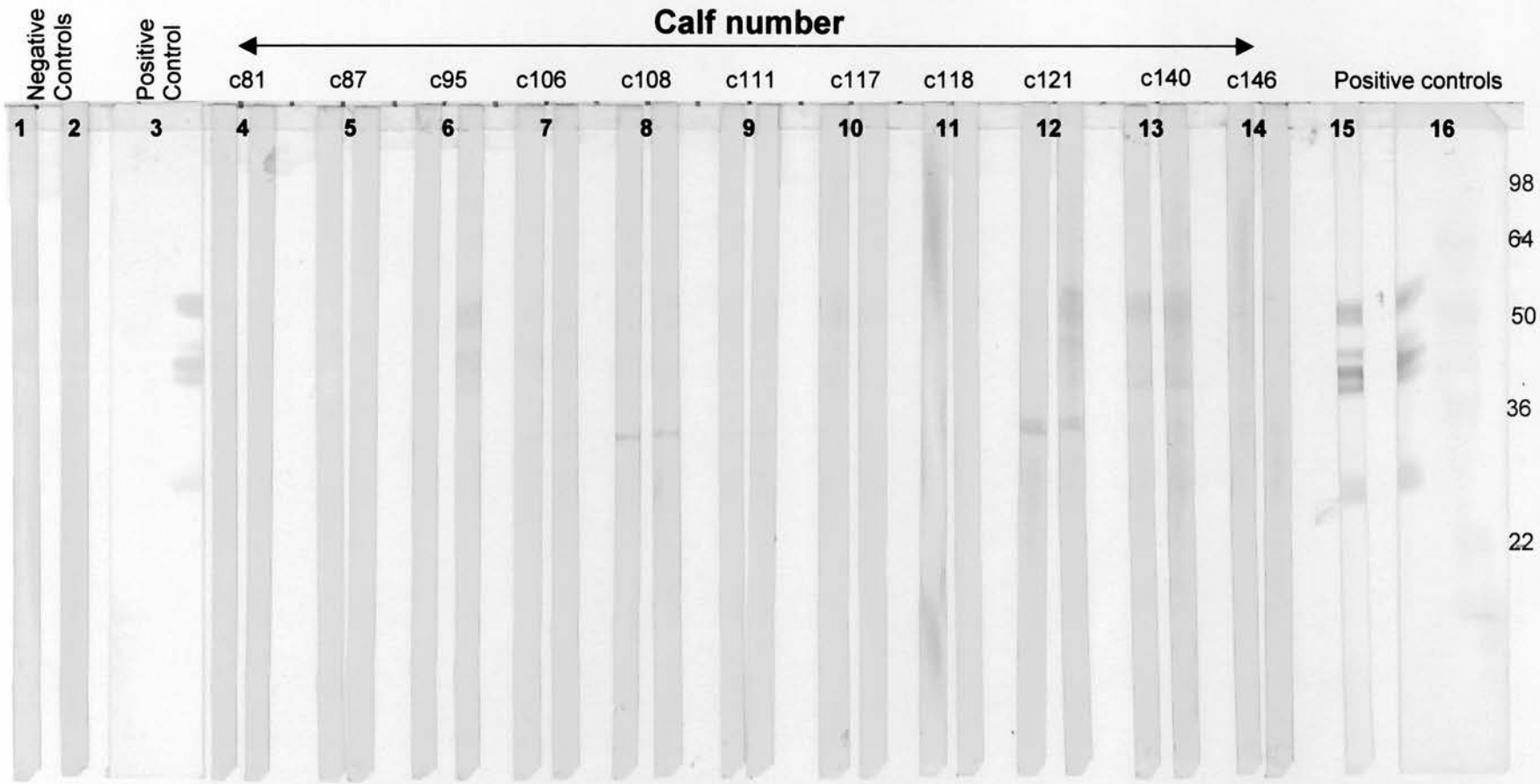


Figure 5.6: The immune response to EspB in 11 experimentally infected calves. Immunoblot showing the anti-EspB IgG status of 11 experimentally challenged calves (lanes 4-16). Each pair of blots represents the pre and week four-post challenge status of each animal. The positive controls (lanes 3, 15 and 16) show the bands of interest. The major EspB/GST band can be seen at just over 50KDa, with a number of smaller proteolytic fragments beneath. The negative controls in lanes 1 and 2 were generated using foetal calf serum and antibody diluent respectively. Neither shows any significant reaction to the EspB/GST fusions.

5.2 The immune response to surface antigens of *E. coli* O157

Outer membrane preparations of a toxin negative *E. coli* O157 (Zap193) (see **Table 2.1** for details) were prepared. Samples of the preparations were resolved by 10% SDS-PAGE, and proteins transferred to nitrocellulose. Nitrocellulose was cut into strips (3mm x 90mm) and used to examine sera from experimentally infected calves for the presence of antibodies to components present on the outer membrane of *E. coli* O157. This experiment used a different set of 12 animals each receiving an oral challenge of 10^9 *E. coli* O157 and monitored for a period of four weeks. Sheep anti-IgG HRP was used to detect antibody antigen complexes on the nitrocellulose. The results of the blots are shown in **Figures 5.7** (complete results can be seen in **Appendix 3**). The animals chosen are representative of the range of responses observed in all the animals examined.

None of the animals' immune responses appear to have been stimulated during the period of experimental infection. In most cases the basal level of antibody (as shown by the pre-bleed sample) is high and no change is observed in antibody levels over the following three weeks. In calves where the basal level is low, for example c310 or c312, there is also no change in the antibody levels over the period of experimental infection.

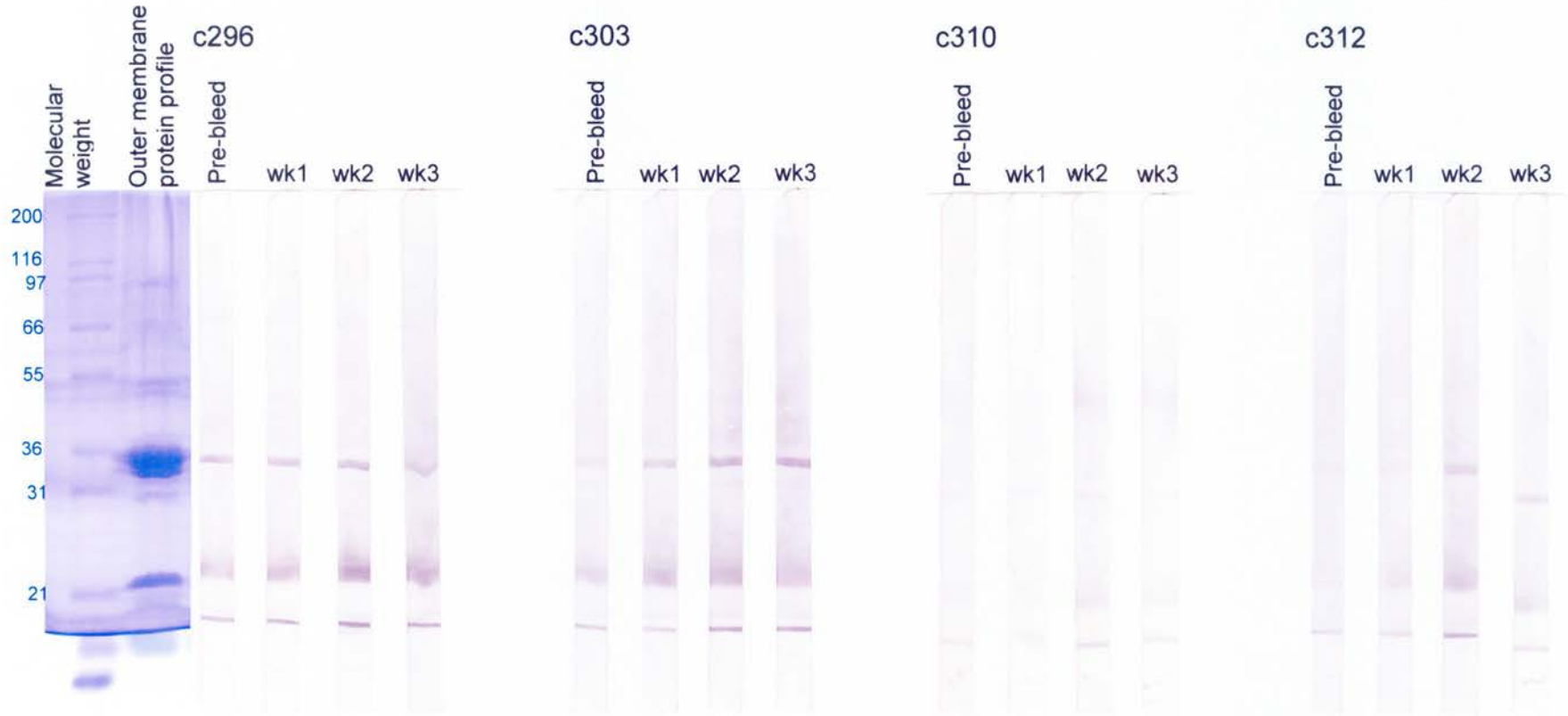
All of the animals appeared to respond to the major proteins visible on the SDS-PAGE gel. The proteins at the 36KDa marker elicit the best response in all the animals. A number of the high molecular weight proteins appear to be

immunogenic in some of the animals but are undetectable in others. Calves c296 and c303 show the best immune response to the high molecular weight material. Both animals respond well to the 36KDa protein(s). There are a number of low molecular weight bands to which the bovine sera react. All the animals appear to react well to components of the unresolved material in the gel front. C310 and c312 show the weakest immune response of all. The pre and week-one sera of c310 failed to show any reaction to the proteins at around 55KDa but weak reactivity can be seen in the week two and three samples. All of the blots appear to have a very blurred background; this is likely due to anti-LPS antibodies. The immune response to LPS will be discussed later.

5.3 The immune response to the secreted proteins of *E. coli* O157.

The secreted proteins of Zap 193, toxin negative *E. coli* O157 were obtained by TCA protein precipitation from the culture supernate. The precipitated proteins were resolved by SDS-PAGE. The proteins were then transferred to nitrocellulose and the immune responses of several experimentally infected calves examined by Western blot. **Figure 5.8** shows the protein profile obtained from the TCA precipitated *E. coli* O157 secreted proteins and the immune response to these proteins in three experimentally infected calves. (Complete results can be seen in **Appendix 3**) The series of three blots shown are representative of the results seen in all the animals tested. There was considerable variation in the range of response as compared with the outer membrane proteins. Most of the proteins seen on the Coomassie

stained SDS-PAGE gel appear to elicit an immune response in most of the animals. The proteins of interest are pointed out on the SDS-PAGE gel and show the translocated intimin receptor (Tir) at 74KDa, the EspB/D complex at around 36KDa and the small filamentous protein EspA at 22KDa. In many of the animals the basal level of antibody (as shown by the pre-bleed status) to some or all of these proteins is high. It appears that the initial level of antibody is unaffected by the period of experimental infection and none of the secreted proteins appears to have stimulated an immune response above the basal level during the experimental period (week-four samples were unavailable for analysis). Calves c313 and c322 show a good response to the EspB/D proteins and also to a slightly smaller protein at about 31KDa. Calf c324 however fails to respond well to these proteins. EspA appears only to have generated a response in c313. The translocated intimin receptor generated an immune response that is just detectable on the blots.



Figures 5.7: Antibody responses to the outer membrane proteins of *E. coli* O157. Outer membrane preparations from a toxin negative *E.coli* O157 resolved by 10% SDS-PAGE and transferred to nitrocellulose for immunoblot. The first blot in each series of four shows the pre-bleed IgG status of each of the four calves (c296, c303, c310 and c312). Each blot thereafter (wk1, wk2 and wk3) represents the immune status at weekly intervals after the initial challenge. Note sera from the fourth week of the experiment were not available. A 10% SDS-PAGE outer membrane protein profile (left hand side) along with the molecular weight of the proteins is shown for reference.

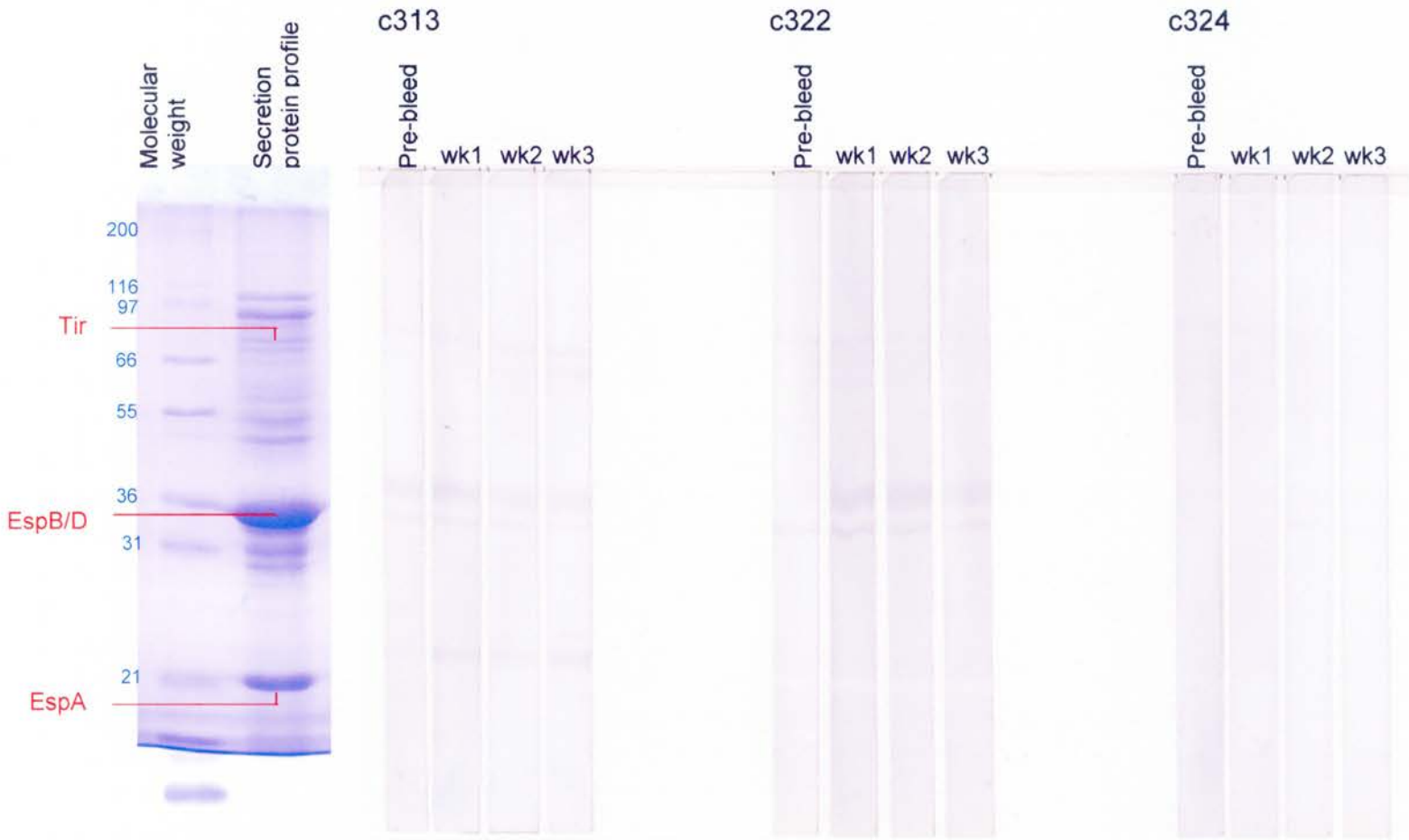


Figure 5.8: Antibody responses to the secreted proteins of *E. coli* O157 in 3 experimentally challenged calves over a three-week period. A series of 12 blots representing the anti-secreted protein IgG status of three animals experimentally challenged with *E. coli* O157 over a four-week period. The first blot in each series of four shows the post challenge (pre-bleed) status of each animal, the three blots thereafter represent the status of sera taken at weekly intervals after the initial challenge. The SDS-PAGE gel (left hand side) is shown for reference along with the molecular weight markers. Proteins of interest are noted at the side. Sera from week four were not available.

5.4 Detection of antibodies reactive to *E. coli* O157 LPS in experimentally infected calves

The LPS of *E. coli* O157 was resolved by 14% PAGE and transferred to nitrocellulose for immunoblot. Nitrocellulose was cut into strips (3mm x 90mm). The nitrocellulose strips were used to detect antibodies reactive to the LPS of *E. coli* O157 in pre-challenge and week-four post challenge sera from a number of experimentally infected calves. Foetal calf serum and antibody buffer were used as negative controls. No reaction to *E. coli* O157 LPS was detected in either. The positive controls were generated using serum from a rabbit previously shown to have antibodies to *E. coli* O157 LPS. The blots (**Figure 5.11**) show reactivity to both the high molecular weight O-antigen repeating units of the LPS and the low molecular weight core region. The response to the O-antigen results in a ladder pattern characteristic of an LPS response (complete results can be seen in **Appendix 3**).

Of the ten animals tested four (calves c87, c108, c117 and c121) had little or no antibody to *E. coli* O157 LPS. The blots representing the pre-challenge and week-four IgG status of these animals appear no different from the negative controls. Two calves c106 and c140 have antibody reactive to *E. coli* O157 LPS in both the pre-challenge and week-four post challenge samples. The level of this response appears to have been unaffected by the period of experimental infection with *E. coli* O157, as the intensity of the bands does not change.

The remaining four calves c81, c95, c111 and c118 have all responded to the LPS of *E. coli* O157. There is little or no antibody present in the pre-challenge sera of these calves, however there is a marked LPS response in each of the animals after four weeks experimental infection. The response is particularly notable in c81. The pre-challenge sample from this animal is devoid of any detectable IgG reactive to *E. coli* O157 LPS. In the week four-post challenge sample, the characteristic ladder pattern denoting an O-antigen response is clearly visible along with a significant response to the core region. The responses in c111 and c118 are less well defined, but still clearly visible. The response in calf c95 is weak and appears to be directed towards the O-antigen only.

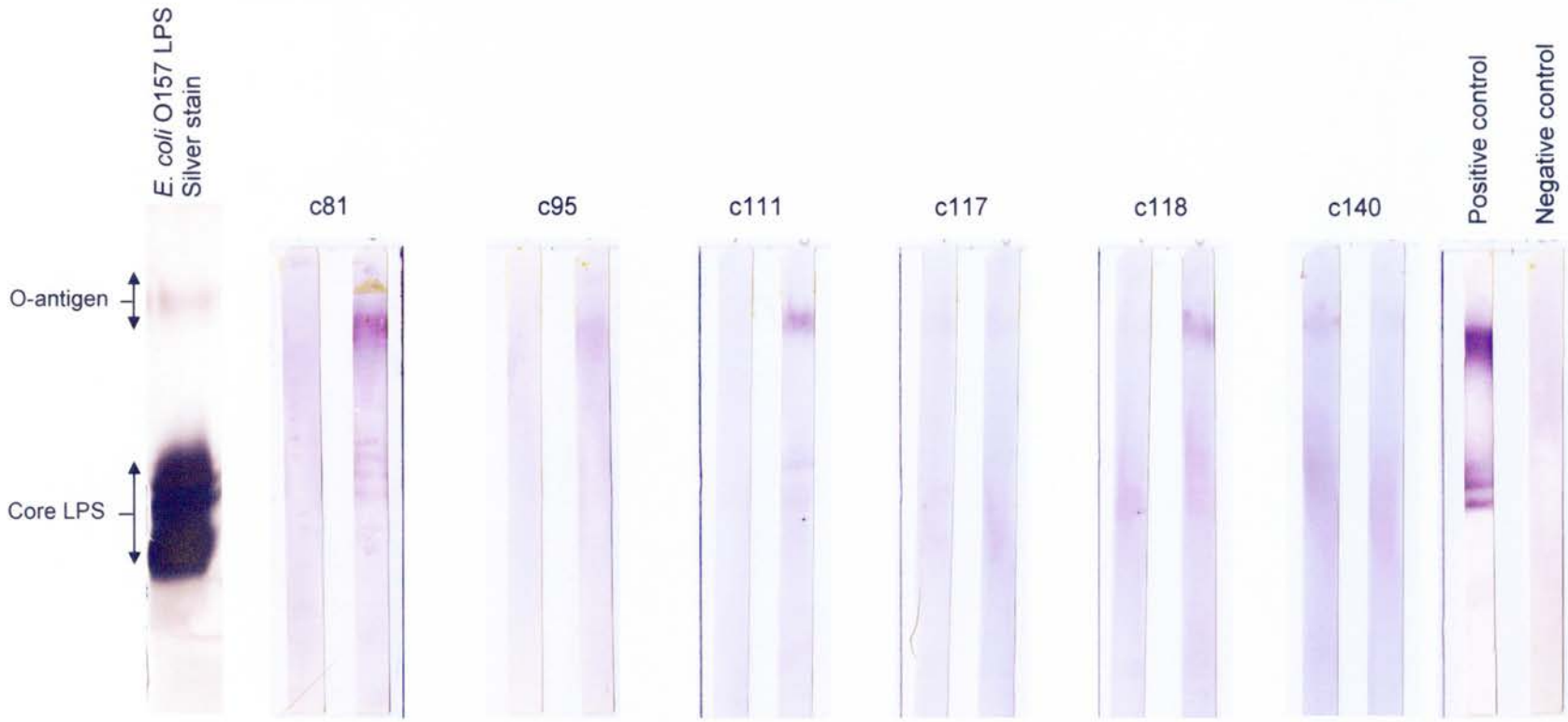


Figure 5.11: The response to *E. coli* O157 LPS in six experimentally infected calves over a four-week period. Each pair of blots shows the post challenge and week four post challenge status of each animal. The positive control (generated with sera from a rabbit known to have anti-*E. coli* O157 LPS antibodies) and the negative control (generated using foetal calf serum) are shown on the right. The silver stained LPS is shown on the left. Note the LPS for silver stain was run on a separate gel.

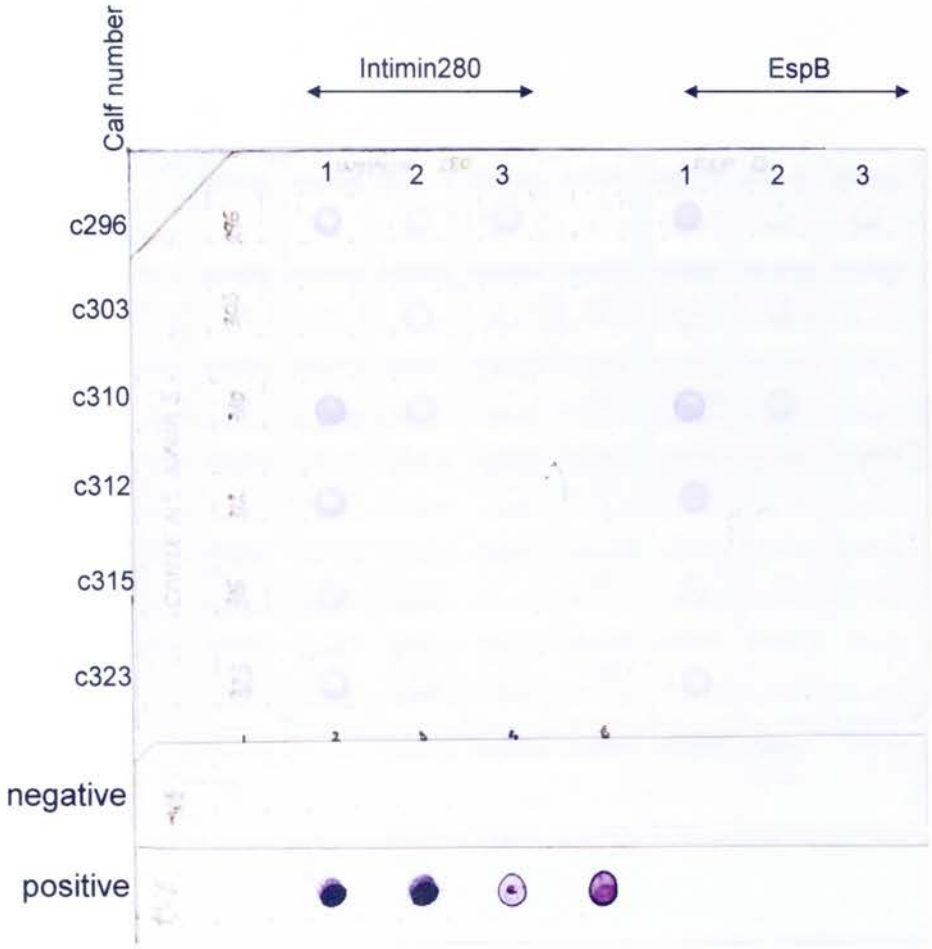
5.5 The mucosal immune response to Int280 and EspB

Various volumes of Int280 and EspB at a concentration of 1mgml^{-1} were spotted on to nitrocellulose to determine the optimal amount for detection. Nitrocellulose was blocked and then goat anti-GST IgG at a dilution of 1 in 1000 in antibody diluent was incubated with the nitrocellulose for 90min at 37°C. The nitrocellulose was then washed twice in TTBS for 10min and then rabbit anti goat-IgG HRP (Sigma) at a dilution of 1 in 10,000 in antibody diluent was added for 90min at 37°C. Colour was developed with HRP colour developer. It was found that there was little difference in the level of reaction between 1μl and 4μl of the Int280 or EspB fusion proteins (data not shown). To reduce the possibility of leakage of antigen across the nitrocellulose it was decided that 2μl of antigen gave a clearly detectable spot without spreading too far through the nitrocellulose. In all subsequent blots 2μl of antigen was used. Faecal samples taken from experimentally infected calves were prepared in category 3 containment facilities. From a starting sample of around 3g (wet weight) bovine faeces, around 3ml of purified, sterile faecal extract was obtained. Faecal antibody extracts obtained from calves c296, c303, c310, c312, c315 and c323 were examined for anti-Int280 and EspB IgA prior to and at weeks one and two post challenge (**Figure 5.10a**). Calves c295, c299, c307, c313, c322 and c324 were examined for anti-Int280 IgA only, prior to and every week for four weeks post challenge (**Figure 5.10b**). Negative controls were generated using foetal calf serum. No Int280 or EspB specific IgA was detected in the negative controls. Positive controls were

generated using an anti-GST antibody and show the presence of a GST-tagged protein on the surface of the nitrocellulose.

Very little IgA was detected in the faeces of these animals. A number of the pre-challenge samples, for example c310 (both anti-Int280 and EspB IgA) and c307 (anti-Int280 IgA) yielded detectable amounts of IgA. All calves in **Figure 5.10a** except c303 had detectable levels of both anti-Int280 and EspB IgA prior to challenge with *E. coli* O157. In general, the level of IgA appears to fall as the experiment progresses, by week-two post infection the level of Int280 IgA has fallen to below the limits of detection in this assay (**Figure 5.10a**). However calf c296 does have a very low level of IgA detectable in both the one and two week post challenge samples. No anti-Int280 IgA was detected in the faecal antibody preparations obtained from the calves shown in **Figure 5.10b**.

a:



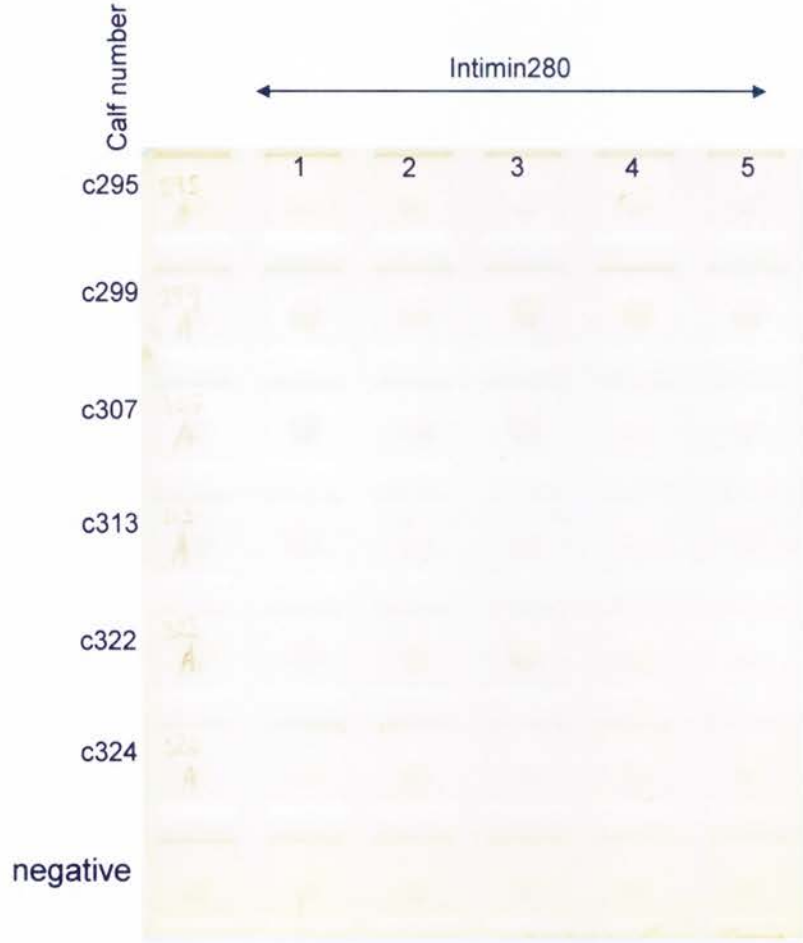


Figure 5.10: anti-Int280 and EspB IgA in the faecal preparations of 12 experimentally infected calves. **a:** The anti Int280 and EspB IgA status of six calves prior to (1), and week one (2) and two (3) after challenge. The negative control was generated using foetal calf serum and the positive control with goat anti-GST IgG. **b:** The anti-Int280 IgA status of six experimentally infected calves prior to (1) and one (2), two (3), three (4) and four weeks (5) after challenge.

5.6 Discussion

A number of antigens from *E. coli* O157 were resolved by SDS-PAGE and transferred to nitrocellulose. The transferred antigens were used to analyse the antibody response of calves experimentally challenged with *E. coli* O157. It was evident that the sera of most of the animals examined contained antibodies to Int280 prior to challenge with *E. coli* O157. Immune responses to Int280 detected before experimental infection did not change as the experiment progressed. A number of calves failed to react to some of the fusion fragments visible below the whole Int280-GST fusion protein band. It is possible that the immunogenicity of the fusion fragments generated during recombinant antigen preparation varies. Adu-Bobie *et al* (1998) were able to identify regions of the C-terminal region of Intimin that possessed greater immunogenicity than others. It is possible therefore that the difference in serum reactivity observed in the experimentally challenged calves to Int280 and associated fragments reflects the immuno-variance of Int280. It is also possible that the loss of certain fragments affects the folding of the fusion protein and therefore the recognition of the protein. Conformational epitopes are both lost and gained during the cleavage of fragments from the fusion proteins, this may lead to cross-reactive responses from antibodies already present in the serum of the experimental calves.

The poor responses to EspB can in part be attributed to the mis-reads and errors within the EspB fusion protein. These errors were noted after sequence analysis of the cloned fragments was obtained and possibly had an effect on the structure of the fusion protein. However a number of sera

obtained from the experimentally challenged calves did produce visible reactions to the EspB-GST fusion (**Figure 5.6**). For example responses to the whole EspB-GST fusion and associated fragments were noted in the week-four post challenge sera of both calves c95 and c121 and in both the pre-and week-four post challenge samples from calf c140. In many cases the addition of the HRP developer solution to the EspB immunoblots led to a high level of background colour that may have masked some of the more faint antibody responses. The intensity of the background was considerably reduced after drying.

In each case there appeared to be no relationship between the level of antibody reactive to either Int280 or EspB and the amount of *E. coli* O157 shed in the faeces of the experimentally challenged calves over the four-week experimental period. For example calf c121, which had a particularly strong response to Int280, shed consistently high levels of *E. coli* O157 over the course of the experimental infection while calf c106 which failed to excrete any detectable *E. coli* O157 had a response to Int280 that by immunoblot appeared as good as that observed in c121. However, there were differences in the responses detected in the sera from both these animals. While it is possible that antibodies to various fragments of Int280 present in the sera from some of the calves may offer protection from colonisation by *E. coli* O157 it is unclear whether the immunoblot data presented here supports such a conclusion. Graphs showing the full complement of shedding results from each of the calves tested may be found in **Appendix 4**.

Outer membrane preparations derived from strain Zap 193 (**Table 2.1**) were used to look for antibody responses to surface components of *E. coli* O157. Responses to many of the surface proteins were noted in all the animals tested (**Figure 5.7**). In all cases antibodies reactive to outer membrane components were observed in sera obtained from calves prior to experimental infection and the response did not change as the experiment progressed. A number of calves, for example c310 and c312, exhibited particularly weak responses to the outer membrane protein preparations throughout the four-week period. Two proteins in particular reacted well with the sera tested. These proteins have an apparent molecular weight of around 35 and 21KDa respectively.

Immune responses to LPS are also evident on a number of the outer membrane preparation immunoblots. For example sera from calf c303 was found to react well with a number of the outer membrane proteins but also showed a considerable background 'smudge' characteristic of an immune response to both the O-antigen and core LPS regions. There appeared to be no relationship between the levels of *E. coli* O157 shed in the faeces of the animals tested and the immune response to the outer membrane components. Animals that showed weak antibody responses, for example calf c310, were found to shed high levels bacteria throughout the whole experimental period, while others with equally low antibody responses shed significantly lower numbers over the same period. By way of contrast, calf c296 had high levels of antibody and shed very low amounts of *E. coli* O157 while calf c303, which shed much higher levels of organisms was found to

have a strong response to the outer membrane preparation. The reason for a lack of correlation between the levels of antibody to outer membrane components and the amount of *E. coli* O157 shed during experimental infection is unclear. It is possible that antibodies detected in this immunoblot assay are cross reactive and are binding to common antigens present on the surface of all *E. coli* and therefore offer no specific protection against *E. coli* O157.

Sera were also examined for responses to the secreted proteins of *E. coli* O157. Most of the animals tested had antibodies reactive to a number of the secreted proteins (**Figure 5.8**), but there was considerable variation in the responses detected. A number of animals, for example calf c324, produced weak responses to the secreted proteins while others such as calf c322, exhibited very strong responses. The reason for the variation in the response is unclear. It is known that different strains of *E. coli* O157 secrete different amounts of proteins (McNally *et al*, 2001), and it is possible that exposure to low and high secretor strains results in the observed differences in antibody responses.

Lipopolysaccharide from *E. coli* O157 was used to detect antibody responses in the sera of experimentally challenged calves. A number of animals were found to have mounted an immune response to LPS during the course of the experimental infection. Sera taken prior to experimental challenge from calves c81, c111 and c118 were found to have little or no antibody reactive to *E. coli* O157 LPS. However sera from the same animals taken four weeks post challenge showed a significant response to LPS. It is unclear whether

this response played a role in protection against colonisation by *E. coli* O157. While no *E.* coli O157 was detected in the faeces of calf c81 throughout the four-week experimental infection, calf c111 shed *E. coli* O157 consistently for the entire four weeks. Thus any relationship between the presence of antibody reactive to LPS and the level of *E. coli* O157 shed in the faeces of experimentally infected calves is unclear.

Attempts to extract IgA from the faeces of the experimentally challenged calves were largely unsuccessful. Despite attempts to prevent the degradation of IgA with the addition of protease inhibitors, delays in obtaining and processing faecal samples may have led to the loss of significant amounts of IgA through proteolytic cleavage.

Much of the ELISA data to be presented in chapter six was based upon results obtained from the immunoblot analysis of the calf sera. As such, the discussion of chapter six is intended to span both chapters five and six as the work is closely related.

Chapter Six

Detection of immune responses to Int280 and EspB by ELISA.

Aims

Immunoblot analysis in chapter five showed qualitatively that a number of experimentally infected calves had detectable levels of antibody to antigens from *E. coli* O157 prior to challenge. The response over time was determined more quantitatively with the use of an ELISA. An attempt to neutralise AE lesion formation on HeLa cell monolayers using bovine serum may help determine a role for the antibody in protection against EHEC associated disease in cattle.

Therefore the aims of this chapter were:

1) To optimise an ELISA for the detection of bovine antibody reactive to the recombinant GST fusion proteins Int280 and EspB.
2) To detect antibody reactive to Int280 in the sera of healthy adult cattle
3) To detect antibody to Int280 and EspB in the sera of calves experimentally infected with *E. coli* O157.
4) To detect IgA reactive to Int280 and EspB in faecal antibody preparations from calves experimentally infected with *E. coli* O157.
5) To use sera from experimentally challenged calves to inhibit the formation of AE lesions.

Results

6.1 ELISA optimisation

6.1.1 Determination of optimal coating concentration.

Recombinant GST was diluted to 100μg, 10μg, 1μg and 0.1μgml^{-1} in coating buffer and 100μl applied in duplicate to the wells of a microtitre plate. Plates were left to coat overnight at 4°C. Goat anti-GST IgG was diluted 1 in 1000 and added to the plate. Antigen-antibody complexes were detected with rabbit anti-goat IgG HRP. Colour was developed and read at 450nm. **Figure 6.1** shows the graph obtained using 10 fold dilutions of GST. At a dilution of 0.1μgml^{-1} the level of detection was very low. GST concentrations of 100μgml^{-1} and 10μgml^{-1} gave maximum OD readings. A concentration of 100μgml^{-1} was in excess of the amount required to coat the plate. It was decided that 10μgml^{-1} was the optimal coating concentration as this was the last dilution before the level of detection began to fall off.

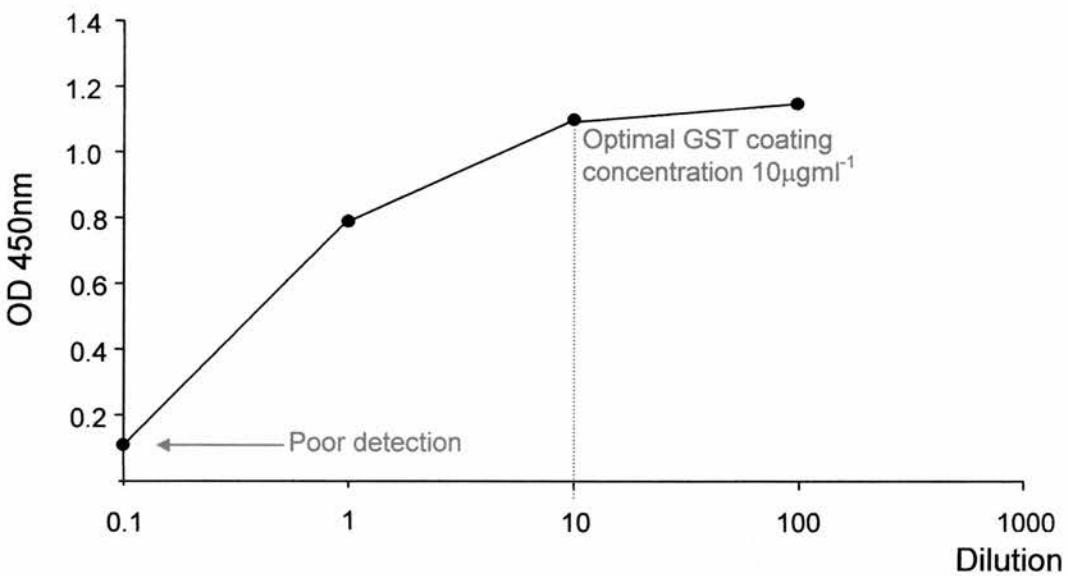


Figure 5.1: Determination of the optimal coating concentration of GST.

6.1.2 Serum titration

In order to ascertain the optimal dilution of bovine serum for the ELISA the bovine sera had to be titrated. The immunoblots (**Figures 5.4 and 5.6**) gave an indication as to which samples had median antibody titres. Samples from calves c95, c106 and c108 were chosen. Samples from calf c95 (pre-bleed and week 4 samples) were the only sera titrated against EspB. Sera was used neat, 1 in 100 and then doubly thereafter until a final dilution of 1 in 6,400. The dilution producing an optical density reading of half the maximum would be considered optimal. This is the dilution at which the ELISA is most sensitive to changes in antibody level. It was found that the half maximum value was obtained at a dilution of 1 in 200 for both the Int280 and EspB titrations. All sera for use in ELISA were diluted 1 in 200.

6.1.3 Conjugate titration.

Sheep anti-bovine Ig HRP (Serotec) conjugates were used throughout to detect antigen antibody complexes. For economical reasons all conjugates were titrated to find an optimal dilution. All conjugates were found to have an optimal dilution of 1 in 20,000.

6.2 Adult bovine sera screen.

To see how the results obtained from the experimentally challenged calves fit in with the healthy adult bovine population, a number of healthy adult bovine samples were screened for antibodies to Int280. Sera were obtained from Dr

Chris Low of the Scottish Agricultural College (SAC) and Dr Michael Pearce of the Wellcome Trust IPRAVE study. The sera represented the immune status of 263 (40 from SAC, 223 from IPRAVE) adult cattle. Microtitre plates coated with GST and Int280-GST were used to detect antibodies to Int280 by ELISA. Each sample was screened for background binding to GST, which was subtracted from the Int280-GST readings to leave values representing the level of antibody specific to Int280 alone. **Figure 6.2** Illustrates the results from the 40 samples obtained from SAC. The graph shows three populations of results. The first population (black) is the range and frequency of occurrence of the optical densities (OD) obtained against the Int280-GST fusion protein. The second population (light grey) is the range of ODs and frequency of occurrence of each OD obtained against the GST protein. The final population (dark grey) is the effective range and frequency of optical densities representing the anti-Int280 IgG status of the animals. **Figure 6.3** shows the range of optical densities representing IgG reactive to Int280 obtained from the 223 serum samples and the frequency with which each range occurred. Many different plates were used in this assay therefore a sample from the first plate with a median optical density value was included on all subsequent plates so that results could be normalised and compared. No comparisons between the data from the IPRAVE sample and SAC samples can be made as the two populations of results were not standardised to each other. Most of the animals tested had little or no antibody (IgG) reactive to Int280. This was particularly notable among the samples from the IPRAVE study with 173 of 223 samples having low/no

antibody. Most of the samples obtained from SAC had levels of antibody that gave OD values in the range 0.2-0.29. In a few of the sera tested very high levels of anti-Int280 IgG were detected.

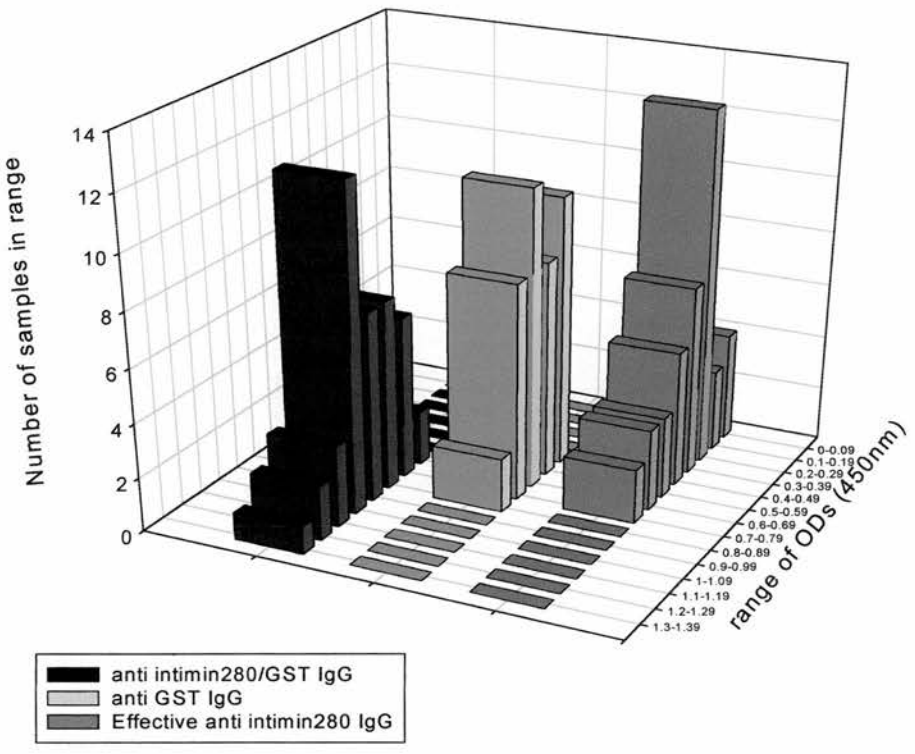


Figure 6.2: Graph to show the range and frequency of optical densities obtained from 40 adult bovine serum samples tested by ELISA for anti- GST, Int280-GST and Int280 IgG.

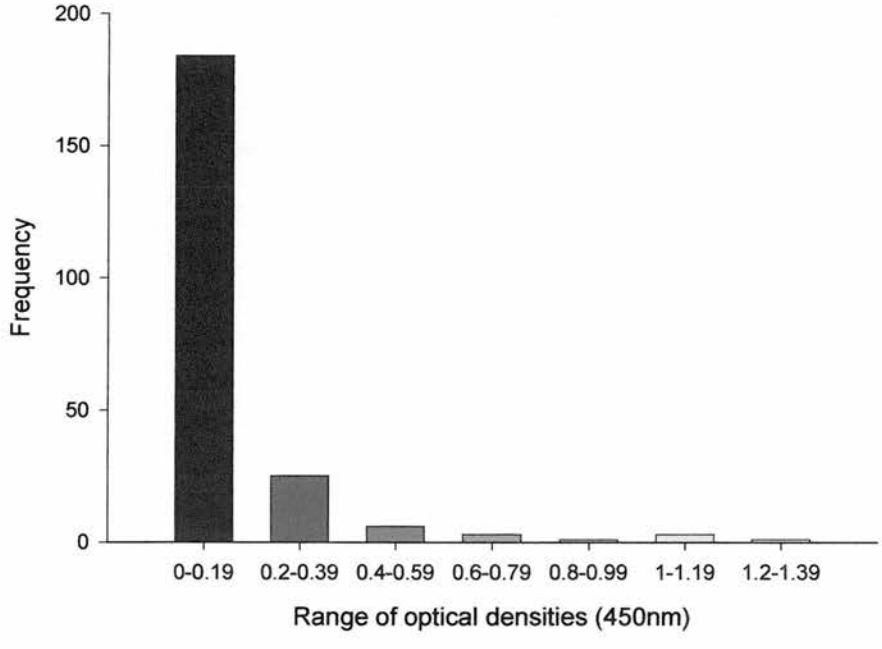


Figure 6.3: Graph to show the range and frequency of optical densities obtained from 223 adult bovine serum samples tested by ELISA for anti-Int280 IgG.

6.3 Detection of antibodies to Int280 and EspB in calves experimentally challenged with *Escherichia coli* O157.

Microtitre plates were coated with recombinant GST, Int280-GST and EspB-GST fusions at 10μgml^{-1}. The GST background was subtracted from the Int280-GST and EspB-GST OD readings leaving an OD for the level of antibody specific for Int280 and EspB. **Figures 6.4-6.11** show examples of the results obtained (all results can be seen **in Appendix 4**).

The amount of *E. coli* O157 shed by each of the animals over the four-week experimental infection period is shown in **Figures 6.4-6.11**. No *E. coli* O157 was detected in the faeces of any of the animals prior to the experiment. Most of the animals shed *E.* coli O157 at some point during the experimental infection, however there was considerable variation in the shedding patterns from each of these animals. For example, calf c106 (**Figure 6.5**) did not shed any *E. coli* O157 while calves c117 and c108 (**Figures 6.6 and 6.8**) shed consistently over the whole period. Calves c87 and c146 (**Figures 6.4 and 6.11**) displayed an enormous amount of variation in the level of *E. coli* O157 shed. On day 14 approximately 3x10^5 cfu *E. coli* O157 gram^{-1} faeces was detected in c87 (**Figure 6.4**) despite no *E. coli* O157 being detected on day three, and relatively low amounts detected on all other days. Calf c108 (**Figure 6.8**) had a similar shedding pattern, with high levels (9x10^3 – 1.2x10^4 cfu gram^{-1} faeces) of *E. coli* O157 detected on days three, 10, 17 and 21 and very low levels detected in the intermediate samples (30-350 cfu gram $^{-1}$ faeces).

The amount of *E. coli* O157 shed by calf c117 (**Figure 6.6**) was consistently low. On days three, 14, 17, 21 and 24, only 1cfu gram^{-1} faeces was detected. Levels were seen to rise on days seven and ten but only to 30 cfu gram^{-1} faeces. The highest level of *E. coli* O157 shedding was exhibited by c121 (**Figure 6.10**). From day three onwards the level shed was high, peaking at approximately 4.5x10^{5} cfu gram^{-1} faeces. Results for this calf only extend as far as day 21 as the animal was put down on day 23 due to a respiratory illness.

The ability of the *E. coli* O157 inoculum to colonise the calves appeared unaffected by the level of antibody present. For example calves that did not shed any *E. coli* O157 were found to have both high and low anti-Int280 or EspB antibody levels. The same was true of animals that were colonised by the inoculum. Of the animals that were colonised by the inoculum, shedding of *E. coli* O157 progressed for approximately one month.

Figures 6.4-6.7 show the level of anti-Int280 immunoglobulin in four experimentally challenged calves over a four-week period. On average the level of anti-Int280 antibody appears not to change. Many of the calves have antibodies to Int280 prior to challenge however calf c87 (**Figure 6.4**) has very little anti-Int280 antibody prior to challenge and the level of antibody does not change as the experiment progress. While the levels of antibody reactive to Int280 were almost identical in the pre-challenge and week four post

challenge samples, small fluctuations in antibody levels were detected in the intermediate samples, although these readings tend to have larger errors associated with them. In all of the animals the level of IgG1, IgG2 and IgM appears low when compared with the total level of IgG.

The EspB antibody status in four of the 11 animals tested can be seen in **Figures 6.8-6.11**.The basal level (the pre-bleed status) of anti-EspB IgG in some of the animals was high, for example calf c95 (**Figure 6.9**) had a high level of anti-EspB IgG prior to challenge with O157. Other animals, for example c146 (**Figure 6.11**) had or very low (or virtually no detectable) levels of anti EspB antibody throughout the experiment. Despite the obvious colonisation and consistent shedding of the *E. coli* O157 inoculum in many of the animals no change in the immune response was noted. The levels of IgG2 and IgM in each of the animals were negligible and no response was noted. It is apparent that experimental infection with *E. coli* O157 failed to stimulate an immune response in any of the animals.

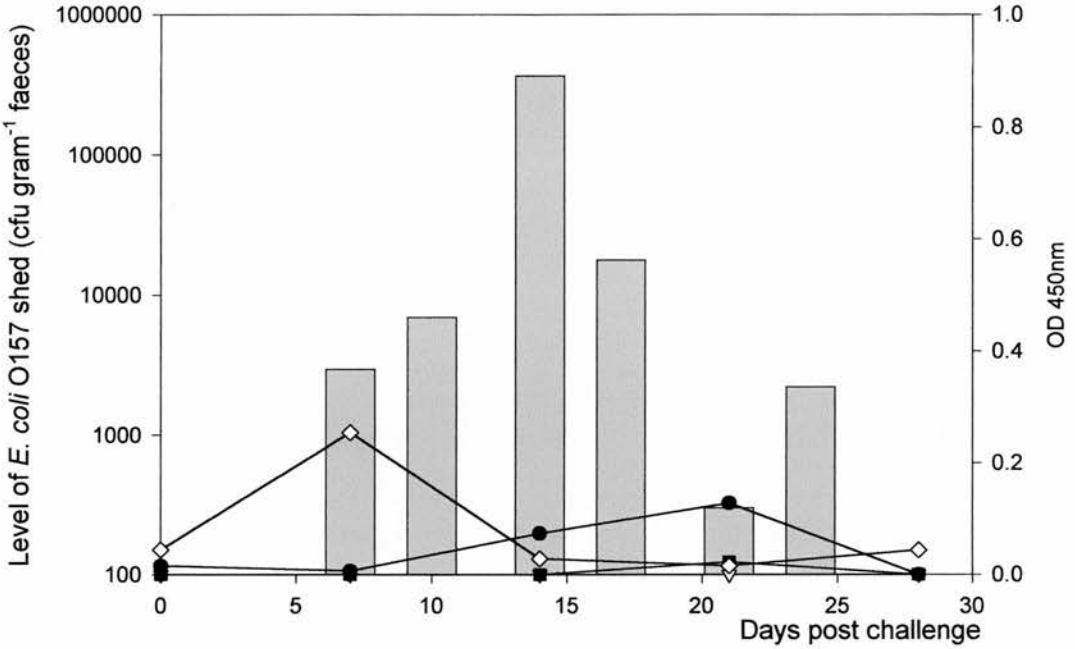
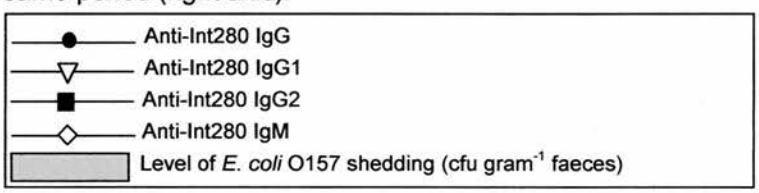


Figure 6.4: Calf c87. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-Int280 immunoglobulin over the same period (right axis).



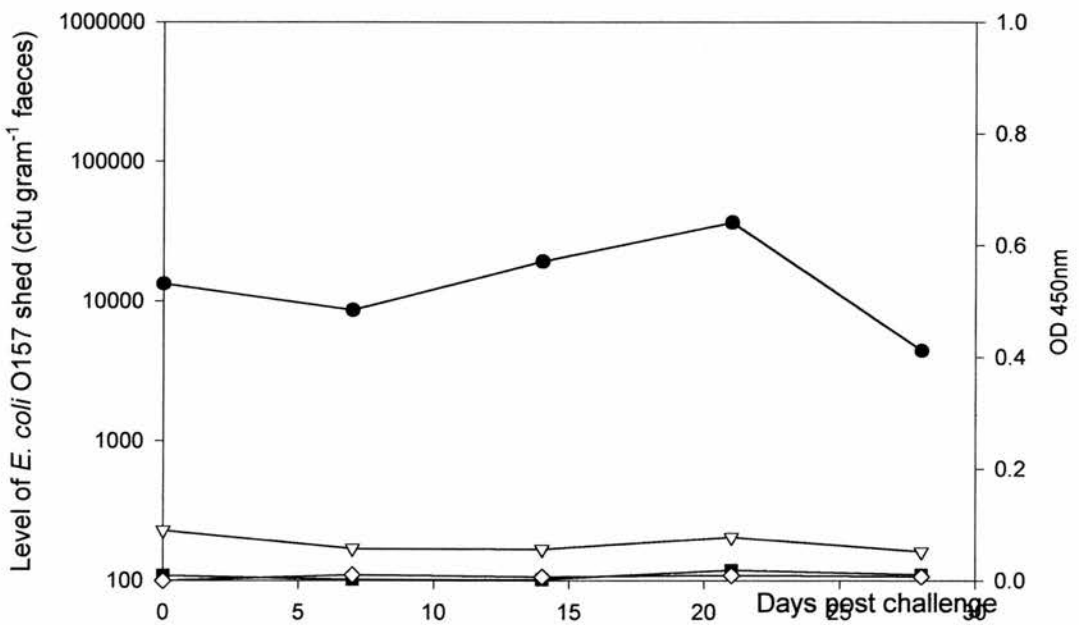


Figure 6.5: Calf c106. Legend as for **Figure 6.4**. This calf shed no *E. coli* O157.

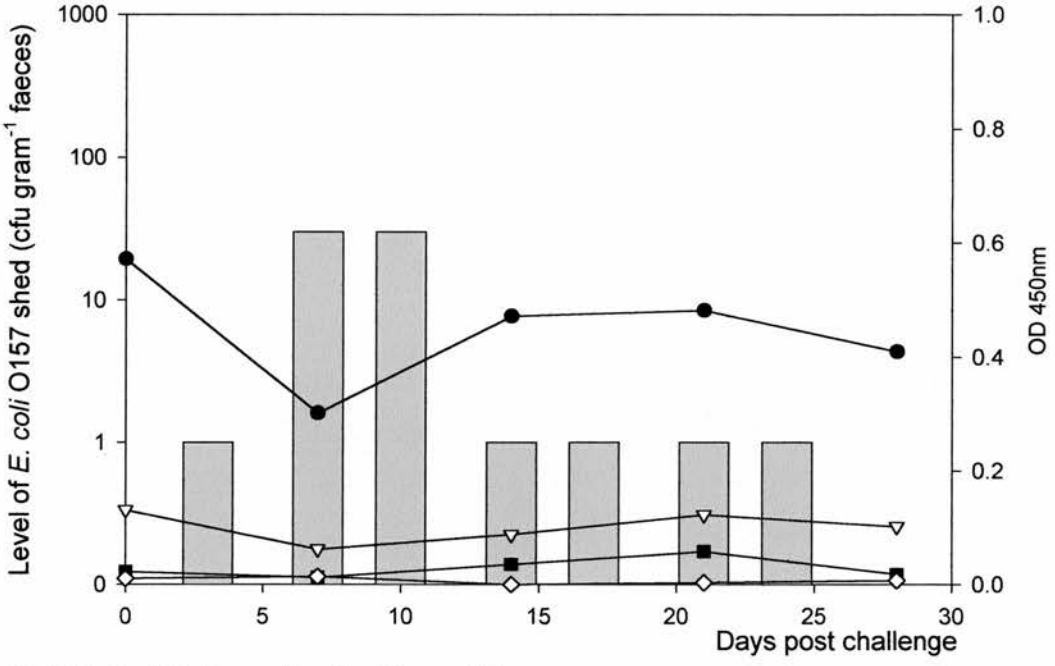


Figure 6.6: Calf c117. Legend as for **Figure 6.4**.

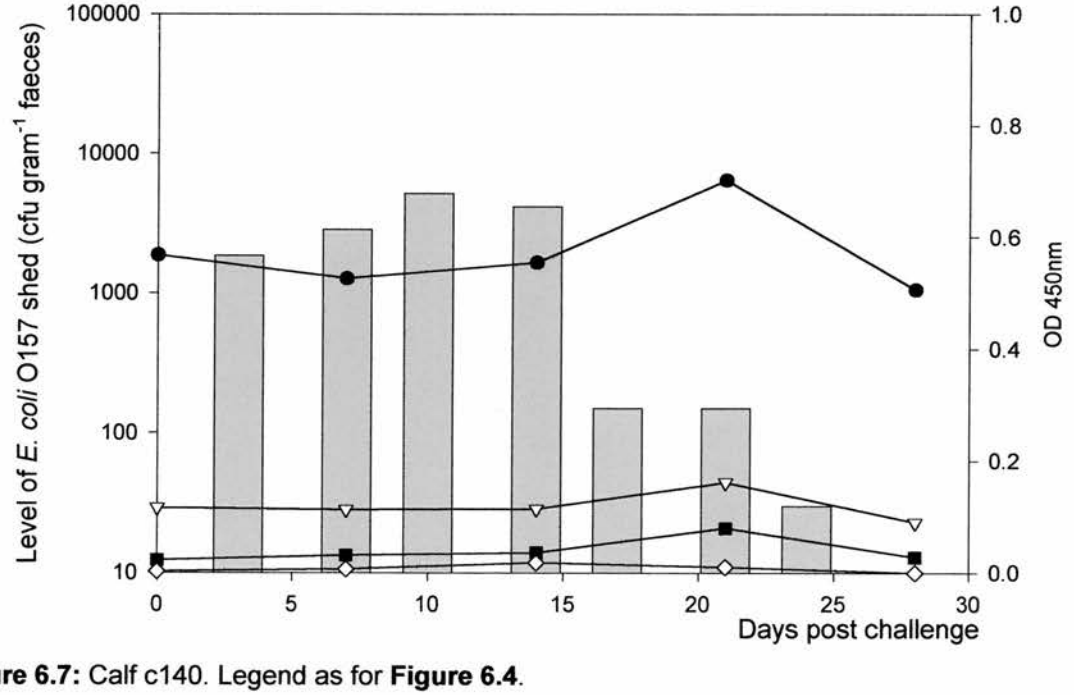


Figure 6.7: Calf c140. Legend as for **Figure 6.4**.

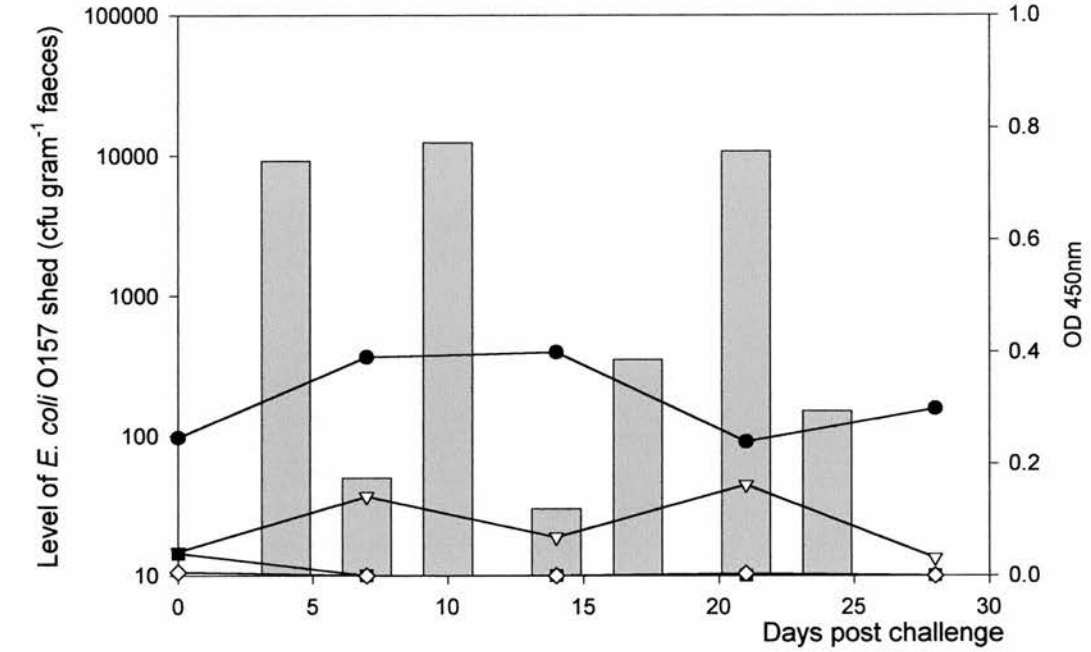
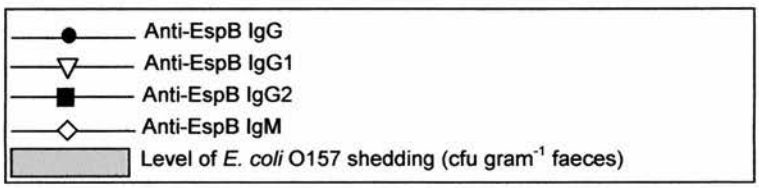


Figure 6.8: Calf c108. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-EspB immunoglobulin over the same period (right axis).



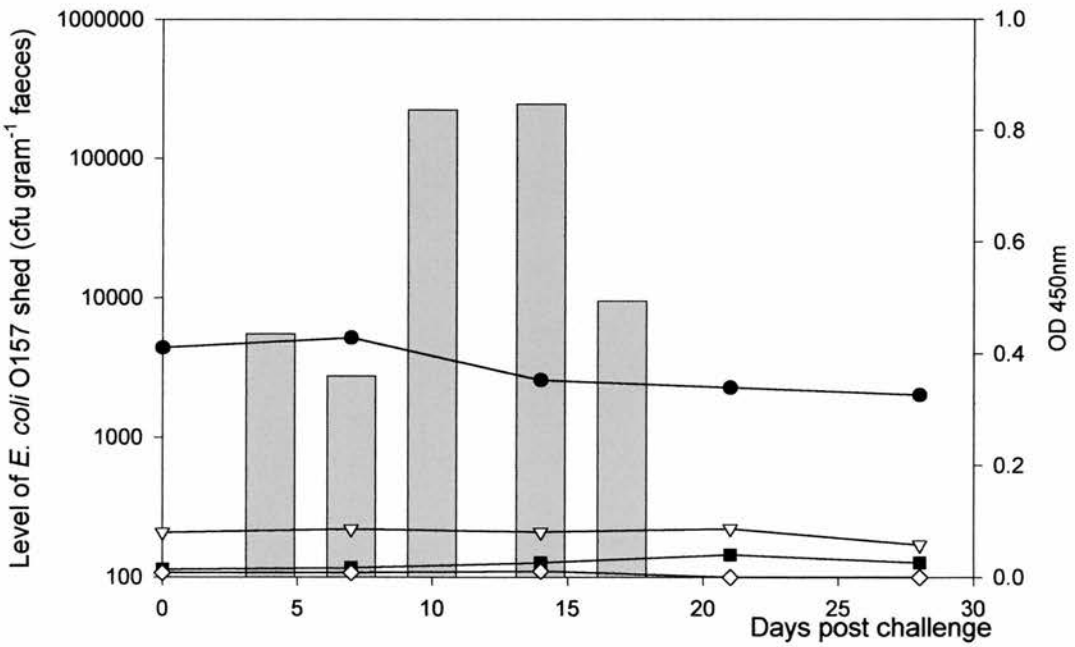


Figure 6.9: Calf c95. Legend as for **Figure 6.8**.

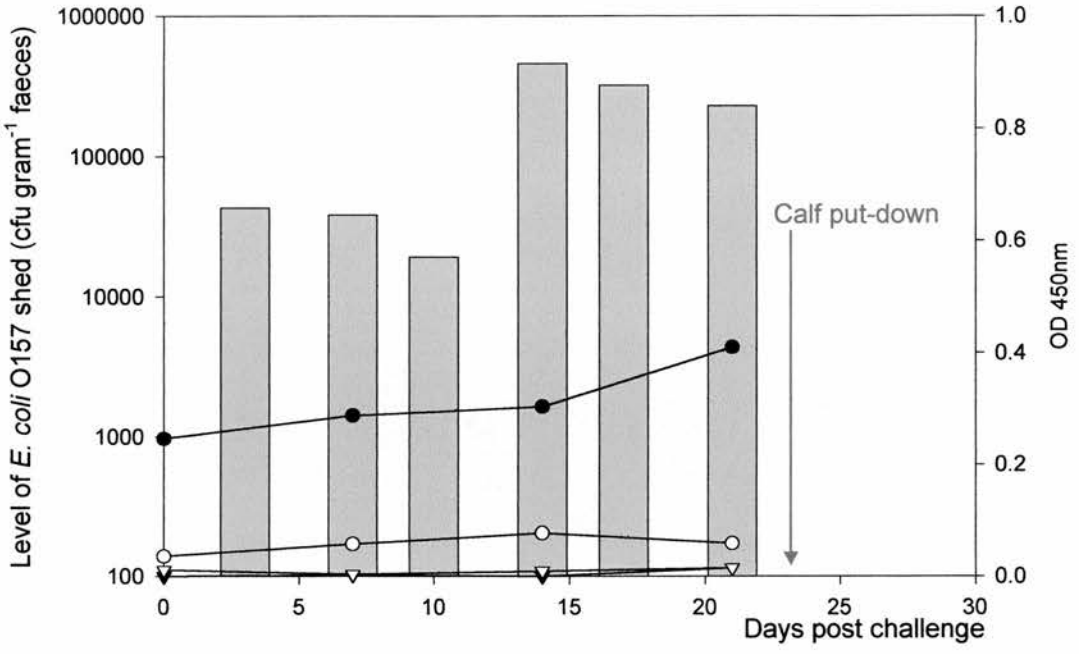


Figure 6.10: Calf c121. Legend as for **Figure 6.8**.

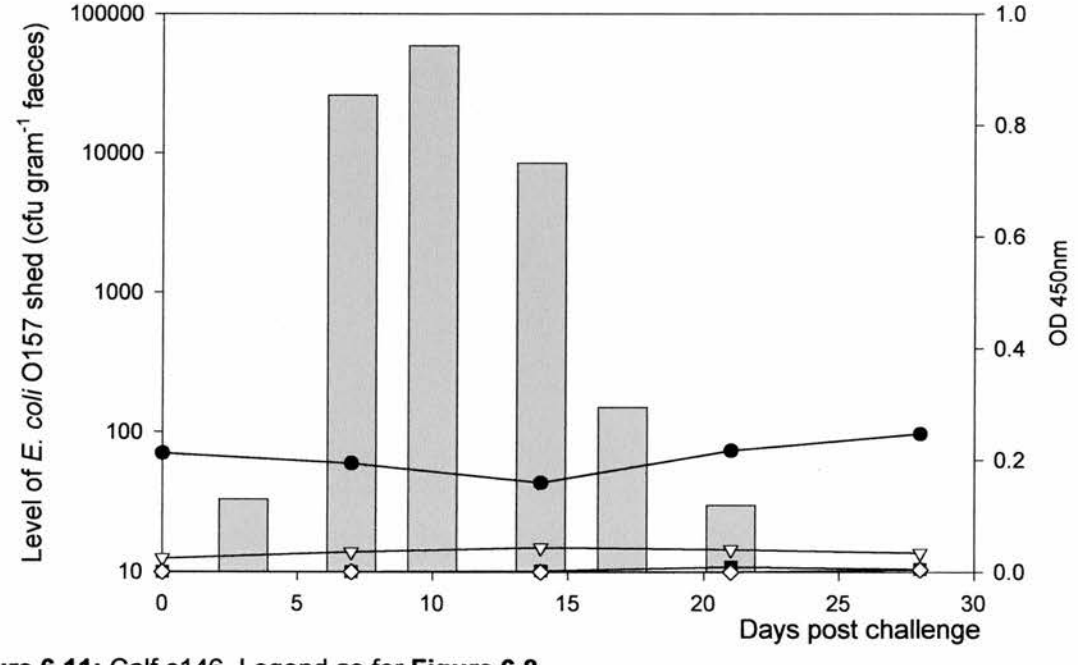


Figure 6.11: Calf c146. Legend as for **Figure 6.8**.

6.4 ELISA to determine the level of specific IgA present in the faeces of experimentally infected calves

ELISA was used to provide an accurate picture of the level of anti-Int280 and EspB IgA in the faeces of the experimentally infected calves over a four-week period.

6.4.1 Determination of the optimal faecal antibody preparation dilution

In order to determine the optimal dilution of the faecal antibody extract to maximise IgA detection, a titration of the extract was set up. Faecal extract diluted 1 in 2 and doubly thereafter to a final dilution of 1 in 8192 was used to optimise the ELISA. Microtitre plates were coated overnight at 4°C with either Int280 or EspB at 10μgml^{-1}. Plates were blocked as described previously. The pre-challenge sample from calf c312 was judged to have given a median reaction by dot blot and was used in this titration experiment. Volumes (100μl) of each faecal extract dilution were incubated with the appropriate antigen for 90min at 37°C. Plates were washed and sheep anti-bovine IgA was used to detect the antigen antibody complexes. Colour was developed and read at 450nm. The titration curve is shown in **Figure 6.12**. From an initial dilution of 1 in 2 the level of IgA detection was very quickly diluted out. At a dilution of 1 in 32 the IgA in the faecal extract had been diluted to beyond the limits of detection. It was decided that the optimal dilution of the faecal extract was 1 in 4 as this allowed maximal detection of any change in IgA titre.

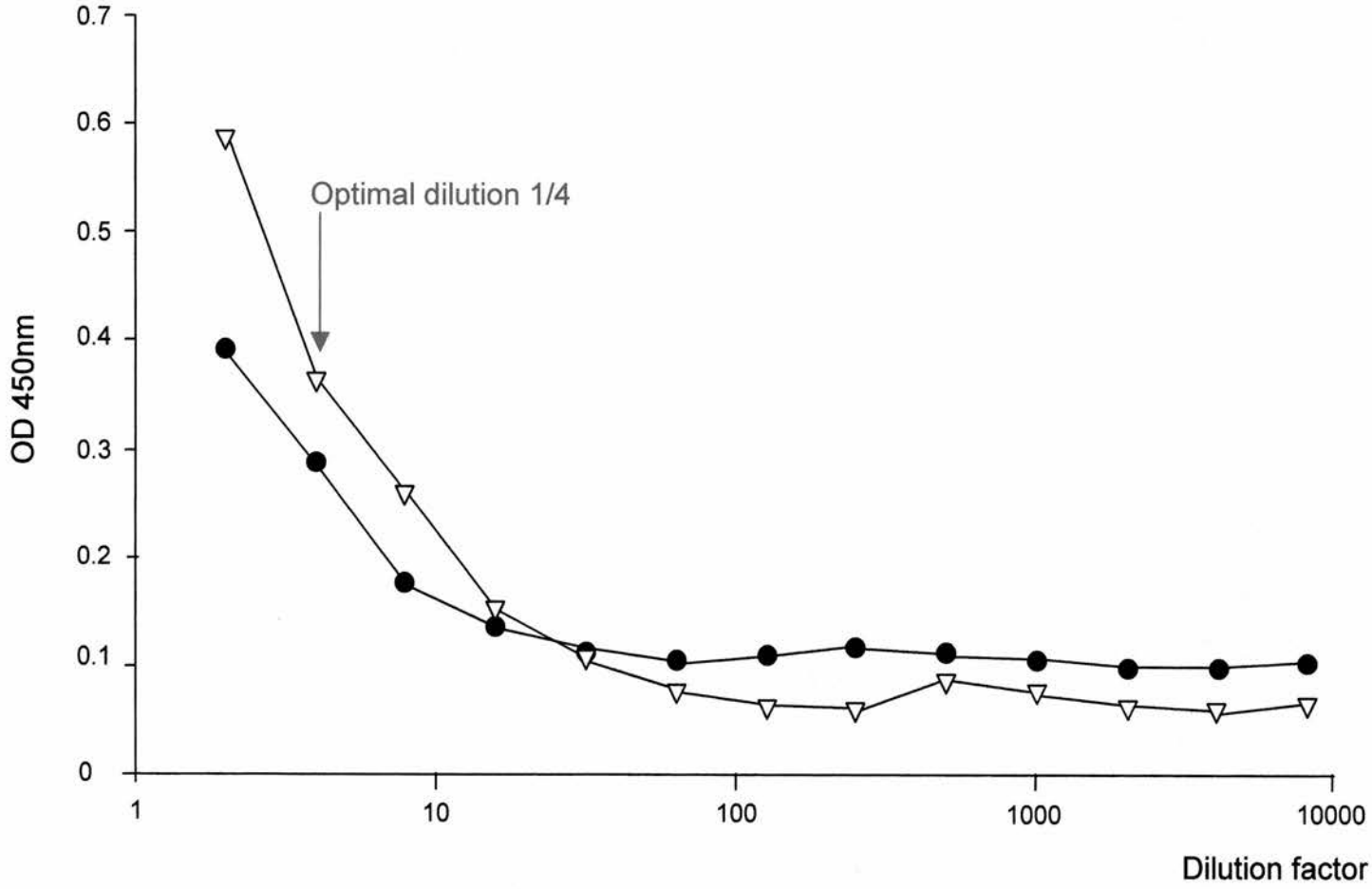


Figure 6.12: Titration of faecal antibody preparations to determine the optimal dilution for IgA detection. Anti-EspB IgA (—●—) and anti-Int280 IgA (—▽—).

6.5 Detection of IgA in faecal antibody preparations from experimentally infected calves.

Microtitre plates were coated with GST and either Int280-GST or EspB-GST at 10μgml^{-1}. Plates were blocked as described previously. Faecal antibody preparations from the experimentally infected calves were diluted to the optimal dilution as determined previously. Volumes (100μl) of each sample were added to the wells of the microtitre plate for 3h at 37°C. An extended incubation time was used to improve detection. Plates were washed and the antigen antibody complexes detected with the use of sheep-anti bovine IgA HRP.

The level of *E. coli* O157 shed from four experimentally infected calves is shown on **Figures 6.13-6.16** (complete results may be seen in Appendix 4). There is considerable variation in the level of shedding from each calf. Calf c299 (**Figure 6.13**) shows a consistently high level of *E. coli* O157 shedding throughout the whole experiment. By day three the level of *E. coli* shed was approximately 4.5x10^4 cfu gram^{-1} faeces, this rose to a maximum of 6x10^5 cfu by day ten. The level of shedding dropped off slightly towards the end of the experiment but was still high. No *E. coli* O157 was detected on day 28. By way of contrast calf c313 (**Figure 6.14**) excreted the highest levels of *E. coli* O157 of any calf, but this was sustained for a period of only ten days. The level of *E. coli* O157 shed peaked on day ten at 1.3 x10^6 cfu gram^{-1} faeces. By day 21 no more *E. coli* O157 was detected. Calf c307 (**Figure 6.15**) shows a similar pattern of shedding with a maximum of 3.6x10^4 cfu

gram^{-1} faeces being detected on day 14, this rapidly falls off and by day 17 no more *E. coli* O157 was detected in this animal.

Figures 6.13-6.16 also show the level of anti-Int280 and EspB IgA in four different animals over a four-week experimental infection. Results shown are representative of all the animals tested (complete results may be seen in **Appendix 4**) There is very little variation in the level of anti-Int280 and anti-EspB IgA during the four-week experimental infection. Levels of IgA detected in the pre-challenge sample did not alter as the experiment progressed. A number of the calves, for example c313 (**Figure 6.14**) had virtually no detectable IgA to either antigen. Generally the level of IgA reactive against either Int280 or EspB was low. Results from the dot blot analysis of these faecal extracts, showed that very little anti-Int280 or EspB IgA was present in these samples and this was confirmed by the ELISA results.

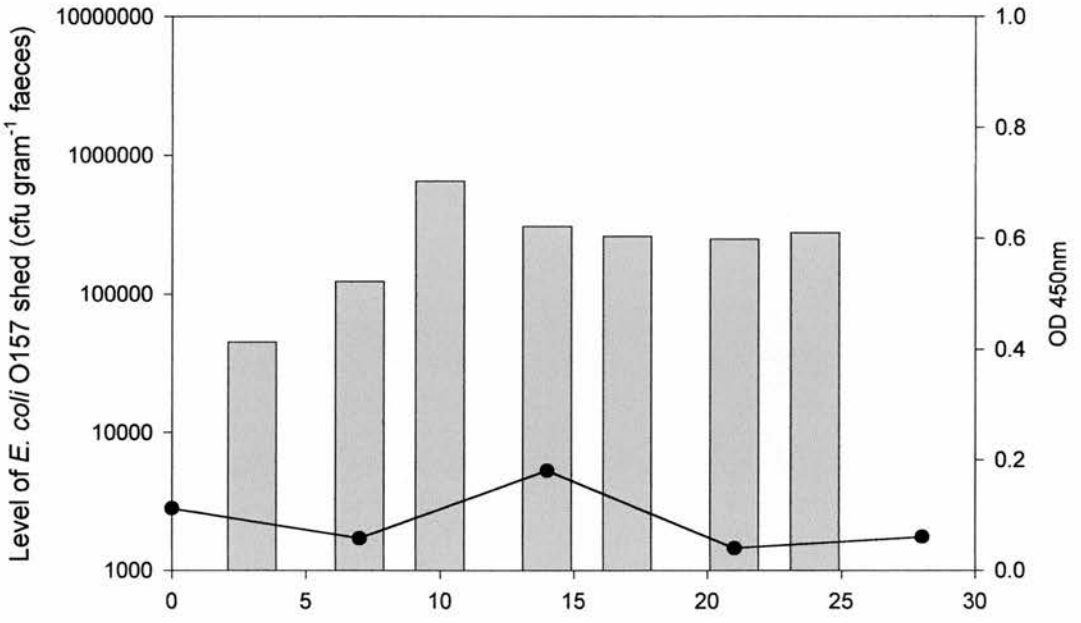
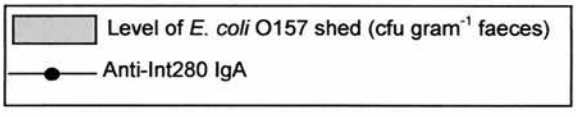


Figure 6.13: The level of *E. coli* O157 shed from an experimentally infected calf (c299) over a four-week period and the level of anti Int280 IgA over the same period.



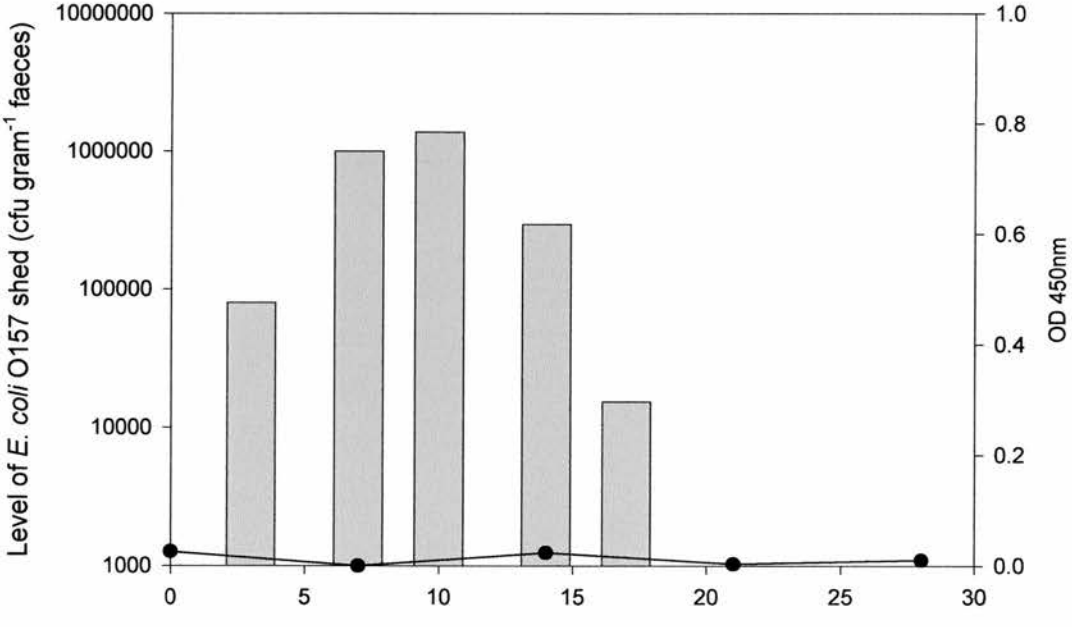


Figure 6.14: c313. Legend as 6.13

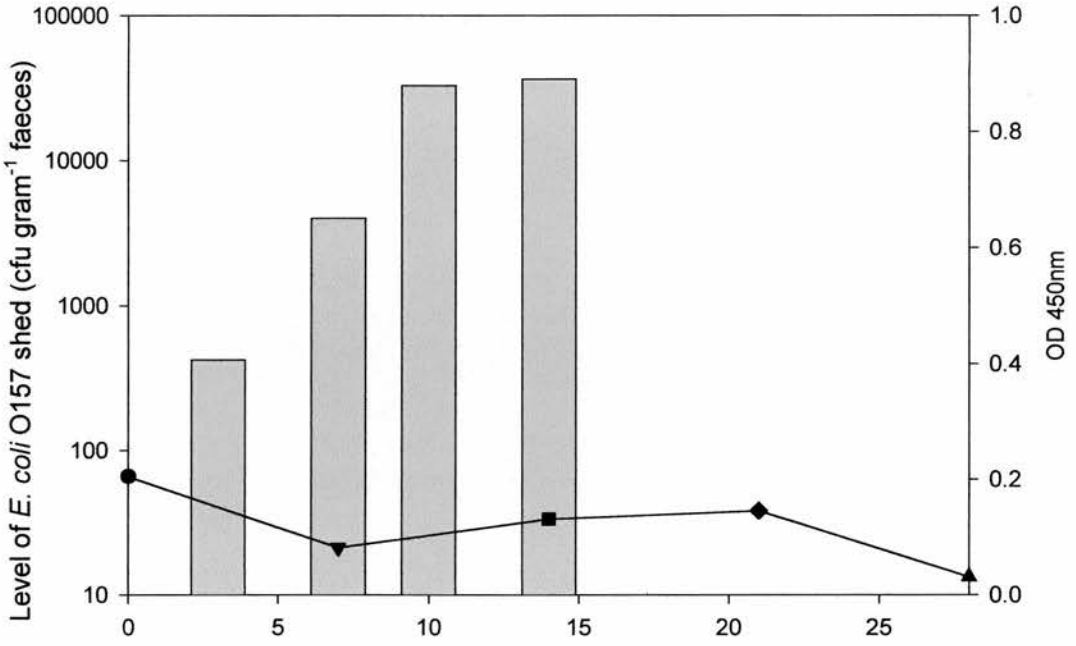
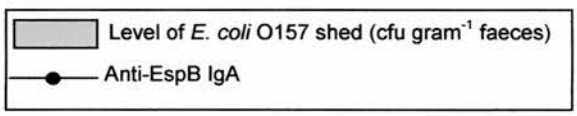


Figure 6.15: The level of *E. coli* O157 shed from an experimentally infected calf (c307) over a four-week period and the level of anti Int280 IgA over the same period.



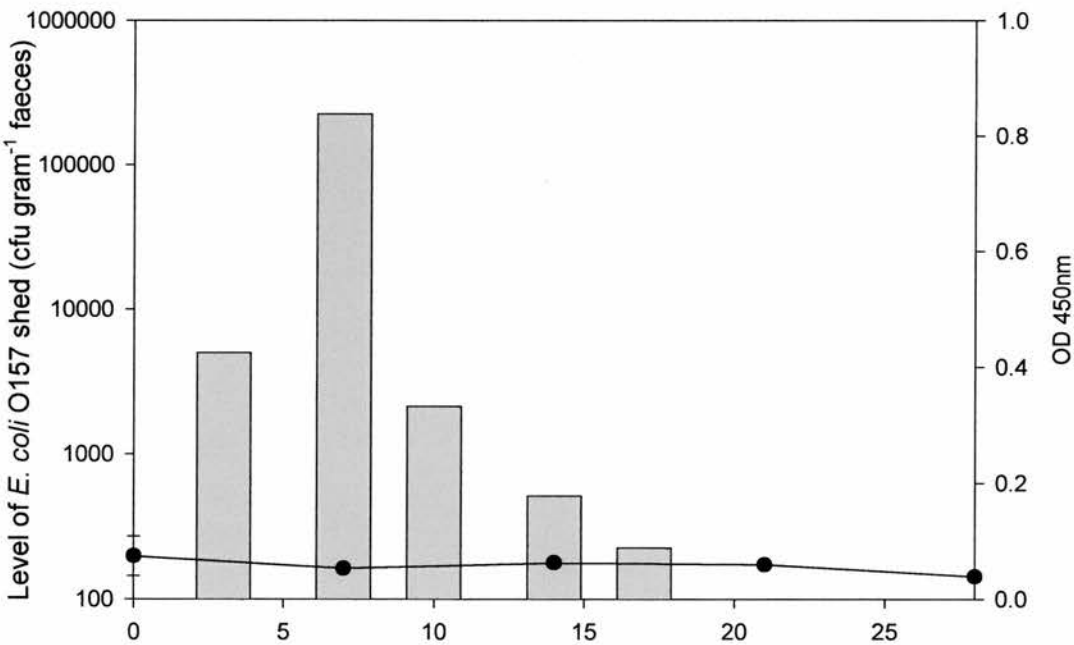


Figure 6.16: c324. Legend as 6.15

6.6 Total immunoglobulin

Both faecal antibody preparations and serum samples from the experimentally infected animals were assayed by radial immunodiffusion (RID) to ascertain the total IgG and IgA content of each sample. Volumes (5μl and 10μl respectively) of serum and faecal antibody preparations were loaded onto each agarose plate. The plates were incubated at room temperature for at least 18h to allow the immuno-precipitation rings to form. A high, medium and low bovine IgA and IgG standard of known immunoglobulin content was assayed along side all the samples. Each animal was found to have a consistent level of immunoglobulin over the four-week period of infection. The IgG content of the bovine serum samples ranged from 890 to 2100mgdl^{-1}. The level of IgA present in the faecal antibody preparations was determined the same way. The level of IgA found in those samples that gave a good reaction by dot blot was below the level of the lowest standard. All the faecal antibody preparations were found to have less than 15mgdl^{-1}. Immuno-precipitation had occurred around the well of each of the samples indicating the presence of very low amounts of IgA. Serum samples from the experimentally infected calves were assayed for serum IgA. The results were similar to those for the faecal antibody preparations in that each sample was found to have an IgA concentration lower than that of the lowest standard (<15mgdl^{-1}).

6.7 The role of anti-Int280 and EspB antibodies in preventing AE lesions.

Bovine serum from experimentally infected calves was used to prevent AE lesion formation by *E.* coli O157 on HeLa cell monolayers. HeLa cells were infected with *E. coli* K12 as a negative control. The phase contrast image (**Figure 6.17a**) is representative of all the fields examined. No adherent bacteria can be seen and fluorescent actin staining (**Figure 6.17b**) with TRITC-phalloidin reveals no actin polymerisation characteristic of AE lesion formation. The merged phase and TRITC images (**Figure 6.17c**) show the cytoskeleton of a normal HeLa cell monolayer. As a positive control HeLa cells were infected with *E. coli* O157, Zap 193. The x100 phase contrast image (**Figure 6.18a**) shows a number of *E. coli* O157 adhered to a HeLa cell. Fluorescent actin staining of this cell (**Figure 6.18b**) reveals areas of dense actin polymerisation. The merged image (**Figure 6.18c**) shows that the actin polymerisation occurs directly beneath the site of bacterial adherence. The *E. coli* O157 bound to the HeLa cell has induced AE lesion formation.

Bovine serum from experimentally infected calves, known to have antibodies to both Int280 and EspB (as determined previously) was pooled and was used to try and prevent AE lesion formation by *E. coli* O157 Zap 193. Bacteria were first cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with pooled bovine serum diluted to 1 in 20 or 1 in 200. Bacterial were then diluted in fresh DMEM supplemented with bovine serum

at the appropriate dilution to give an effective infectious dose of 1 cfu HeLa cell^{-1}. The addition of bovine serum appeared to have no effect upon the ability of *E. coli* O157 Zap 193 to form AE lesions (**Figure 6.19**). Any micro-colonies observed under phase contrast were large (**Figure 6.19b**). Observation under fluorescence showed that the micro-colonies and had formed large numbers of AE lesions beneath the site of adherence (**Figure 6.19c**). In both the positive control and inhibition assay areas of the HeLa cell monoloayers were devoid of AE lesions, this tended to correspond to those regions that were totally confluent. AE lesion formation was better observed in areas of partial confluence.

Figure 6.17: x100 images of a section of a HeLa cell monolayer infected with *E. coli* K12 as a control. **a:** x100 phase contrast image showing *E. coli* K12 infected HeLa cell monolayer. **b:** Fluorescent actin staining of the HeLa cells with TRITC-phalloidin (no actin polymerisation is visible). **c:** Merged phase and FAS images showing the cytoskeletal arrangement of the HeLa cells.

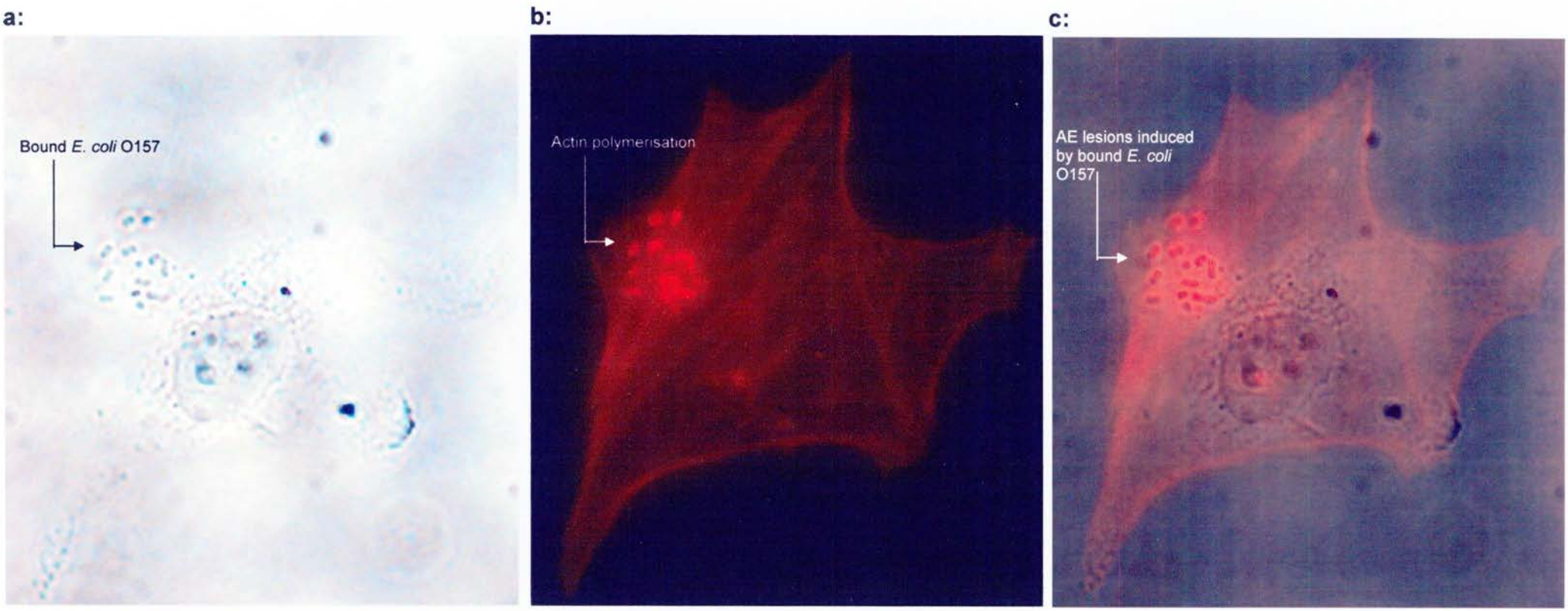


Figure 6.18: AE lesion formation by *E. coli* O157 Zap 193 (see **Table 2.1**) on a HeLa cell. **a:** x100 phase contrast image showing HeLa cell and bound *E. coli* O157 micro-colony. **b:** Fluorescent actin staining of the HeLa cell with TRITC-phalloidin showing dense actin accumulation. **c:** Merged phase and FAS images showing actin polymerisation beneath the site of bacterial adherence.

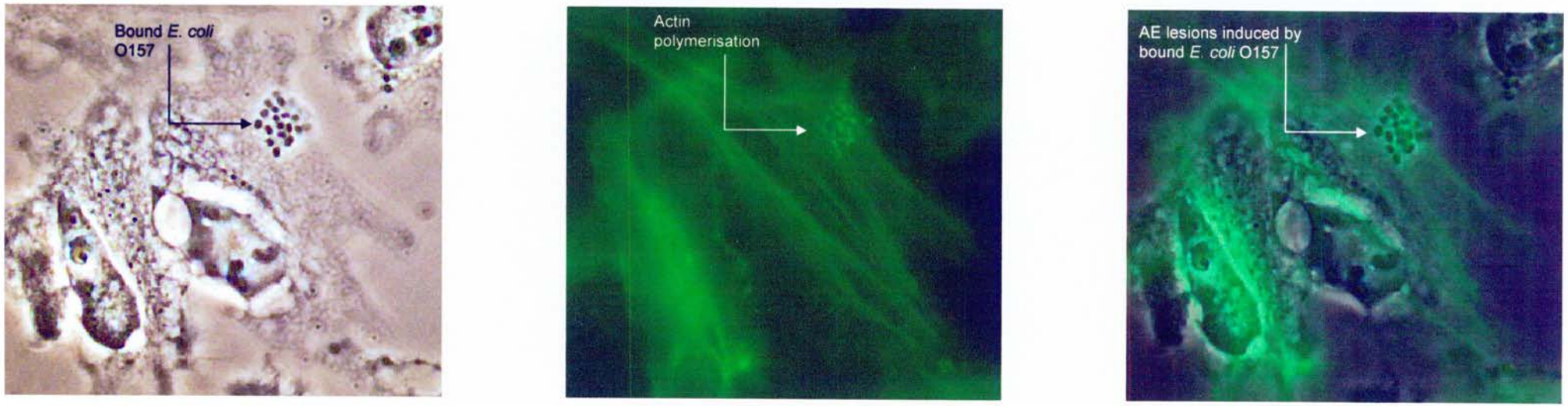


Figure 6.19: Inhibition of AE lesion formation by *E. coli* O157 Zap 193 (see **Table 2.1**) with pooled bovine serum from experimentally challenged calves. **a:** x100 phase contrast image showing *E. coli* O157 micro-colony bound to HeLa cell. **b:** Fluorescent actin stained image (FITC–phalloidin) showing dense actin accumulation. **c:** Merged images showing that actin polymerisation occurs directly beneath the site of bacterial adherence.

6.8 Discussion

A number of calves were experimentally challenged with an oral dose of 10^9 cfu *E. coli* O157 via a feed tube straight to the rumen. Over a four-week period the level of *E. coli* O157 shed in the faeces of each animal was monitored. Blood and faecal samples were taken so that the level of antibodies to a number of surface and secreted components could be examined over the same four-week period. ELISA and Western blot were used to detect immune responses to a wide range of antigens.

The level of *E. coli* O157 shed from these calves over the four-week experimental infection was highly variable. A number calves (c85 and c106) failed to shed any *E. coli* O157, while others shed consistently high levels throughout the experimental period. This is consistent with the findings of others (Cray & Moon, 1995, Besser *et al*, 1997). Brown *et al* (1997) reported that diet might affect the level of shedding observed. While no definite relationship between diet and shedding was described, the level of *E. coli* O157 shed from a number of calves increased when food was withheld. It is likely that diet and other factors, for example age and health, influence the level of *E. coli* O157 shed from experimentally infected calves, and that the influence of these factors varies between calves.

Work by Besser *et al* (2001) looked at the potential for calves experimentally colonised with a low dose *E. coli* O157 inoculum, to infect other calves (*E. coli* O157 negative) in the same isolation pens. By day 21 post-inoculation,

all calves, including those previously uninfected were excreting the challenge strain. This may explain why the shedding figures from some of the calves can dramatically increase during the course of the experimental infection.

The generation of recombinant EspB and Int280 allowed the detection of bovine antibody specific to these proteins. In each case the background level of antibody reactive to GST had to be considered and subtracted from the recombinant fusion protein readings. It was found that most of the animals examined had detectable levels of antibody that bound to GST. Analysis by western blot showed that the level of antibody binding to GST was very low. ELISA however demonstrated that the response was far more pronounced in the sera from the latter half of the experiment. The reason for this is unclear; indeed the reason for any antibody showing reactivity to GST is also unclear. Work by Li *et al* (2000) used GST tagged Int280, EspB, EspA and Tir to detect antibodies in the sera of patients infected with *E. coli* O157. Immunoblot analysis of the sera showed that there was a considerable response to these recombinant antigens but no significant response to the GST alone. Glutathione *S*-transferases belong to a group of detoxification enzymes (Sommer and Nimtz, 2001). The GST used in the pGEX gene fusion system originates from the parasite *Schistosoma japonicum* (Smith and Johnson, 1988). It has been postulated that the GST proteins may offer parasites the ability to modulate the host immune system (Sommer and Nimtz, 2001). If true, GST proteins could induce an immune response in animals exposed to such parasites. This may explain the low level affinity of

some bovine antibodies to GST. No bovine anti-GST IgG2 or IgM was ever detected and the level of IgA from the faecal preparations reactive to GST was also low. It is possible that the IgG and IgG1 conjugates used, had some cross reactivity to the GST component of the protein fusions. This however does not explain the apparent increase in anti-GST titre in the latter half of the experiment. One interesting observation, particularly in the Int280-GST ELISAs was the variation in the negative controls. The background to GST alone was consistently higher than the background to the Int280 fusion protein (and to a lesser extent the EspB fusion). It is thought that the fusion of either Int280 or EspB to the GST may occlude an epitope to which some of the bovine antibodies react. It is important to remember that when GST alone is used it is an entirely different protein in terms of conformation, to a GST fusion protein.

There are therefore a number of problems associated with the subtraction of the GST background from the fusion protein results to obtain a reading for the level of antibody reactive specifically to Int280 or EspB. Firstly, owing to the fact that both the GST and GST fusions bound to the purification columns with reasonable affinity, we can assume that the active site of the enzyme must at least be in the correct conformation. It is however impossible to predict the shape of the remainder of the protein. Any change in conformation may have a dramatic effect upon the immunogenicity of the protein. Despite this however it is important to remember that both ELISA and immunoblot rely on a degree of denaturation to either coat (ELISA) or

resolve (immunoblot) proteins. In which case some of the epitopes will be lost. It is entirely possible that antibodies detected by these methods are not in actual fact the antibodies that offer protection *in vivo*.

It was also noted that there were a number of contaminating proteins eluted from the affinity purification columns. Since all the fusion proteins were generated in the same strain of *E. coli* it was assumed that most of the contaminating proteins would be the same in each case and therefore would have a negligible effect upon overall results.

Although by titration the optimal level of coating was determined as being $10\mu gml^{-1}$ it was difficult to decide how to equilibrate the level of GST. While the level of total protein coated on the plate would be the same ($10\mu gml^{-1}$), the amount of GST would not be. The fusion proteins can be considered as whole GST molecules with EspB or Int280 fragments of variable length attached, hence the multiple band profiles of each fusion protein as visualised by SDS-PAGE. In effect the level of background subtracted from the fusion protein readings may actually be too high. To counteract this the GST could have been coated at half the concentration ($5\mu gml^{-1}$) and the fusion protein at the dilution deemed optimal ($10\mu gml^{-1}$). This may have led to there being more GST in those microtitre wells coated with the GST fusion protein, and hence too little GST background would have been subtracted. So long as the method used was consistent and all results were treated the same way any GST aberrations in the readings would be removed.

As mentioned previously much work has been done using recombinant antigens from *E.* coli O157, the favoured method being the use of expression systems that lead to the production of *His*-tagged rather than GST tagged proteins. Such a system employed here may have significantly reduced the level of background observed.

Sequence analysis of the recombinant antigens revealed that the EspB protein had been generated with two errors and without a stop codon in the correct position. Instead the stop codon upon the pGEX-4t_2 vector provided the signal to stop translation. As a result the recombinant EspB protein was generated with an extra sequence of amino acids not present in the native protein. The two Taq polymerase induced errors resulted in the substitution of a glutamine residue for a glycine at position 17, and a serine residue for a threonine at position 196. These errors may have affected the conformation/antigenicity of the protein and may explain the low level of anti-EspB antibody detected in the bovine sera.

Experimental infection with *E. coli* O157 appears to have little effect upon the antibody response to the various membrane and secreted proteins. Many of the calves prior to challenge had detectable levels of antibody (IgG/IgG1) to these proteins. The period of infection failed to have any effect on the level of this response. Other calves appeared naïve to these proteins prior to

challenge, in these animals the experimental infection failed to generate an immune response to any of the proteins examined.

The use of ELISA allowed a more detailed examination of the immune status of these animals throughout the experimental infection. The levels of all the bovine immunoglobulin classes were examined using this technique. A screen of some 260 adult bovine serum samples revealed that in the majority of cases the level of antibody to Int280 is very low. Of the 223 samples obtained from Dr Michael Pearce, approximately 180 were found to be negative for anti-Int280 IgG. Whether or not these animals had ever come into contact with *E. coli* O157 is unclear. Most of the calves used in the experimental infections had detectable levels of antibody to Int280 prior to challenge. This raises the question, why do adult cattle have very low or no anti-Int280 antibody when most calves have detectable levels?

It is possible that the regulation of the LEE pathogenicity island is such that specific environmental conditions are required to bring about expression of the LEE antigens. Studies by Woodward *et al* (1999) showed that a bovine *E. coli* O157:H7 isolate was able to extensively colonise the gastrointestinal tract of gnotobiotic calves. It was noted though that the bacteria were not adhered to the mucosa and no AE lesions were detected. They postulated that colonisation of the gastrointestinal tract of these animals by the *E. coli* O157 strain used, was achieved through an alternative mechanism. In contrast, Dean-Nystrom *et al* (1997) did observe AE lesion formation

indicative of intimin expression, in neonatal calves (<12h and 30-36h) experimentally infected with two different *E. coli* O157 strains. Woodward *et al* (1999) commented that subtle differences between strains yet to be defined might account for the varying ability of *E. coli* O157 to cause disease in neonatal/gnotobiotic calves.

McNally *et al* (2001) were able to show that growth media affected the production of LEE antigens. It is possible that the lack of commensal organisms in gnotobiotic/very young calves has a dramatic effect upon the composition of the gastrointestinal secretions, which in turn could affect the regulation, and production of a number of *E. coli* O157 virulence determinants. If the expression of antigens such as Intimin is affected by such host factors, changes in the gastrointestinal tract as the calf matures may explain the lack of a detectable Int280 response among adult cattle.

Calves acquire all their maternal immunoglobulin from the mother's colostrum; the neonate does not acquire maternal IgG through a placenta, as human beings. It is vitally important that the calf receives the colostrum as soon as possible as in the first few hours the wall of the gut is permeable to large molecules and allows immunoglobulin through. Lissner & Schmidt (1996) showed that bovine colostra contained antibodies reactive to *E. coli* O157 proteins such as verotoxin and haemolysin. In a similar study by Crivelli *et al* (2000) human colostra was investigated and found to contain antibodies reactive to EspB, EspA and Intimin. The passage of bovine

immunoglobulin from cow to calf via the colostrum could provide the antibody that was detected in the pre-challenge samples of most of the calves used in the experimental infections.

Work by Dean-Nystrom *et al* (1997) showed that the ingestion of colostrum by calves prior to experimental infection with *E. coli* O157:H7 failed to prevent colonisation and AE lesion formation. However it is possible that the inoculum used in this study (10^{10} cfu) was too large to properly assess the effects of colostrum. In another study Dean-Nystrom *et al* (2002) were able to show that three pregnant swine vaccinated with purified Intimin mounted significant serum and mucosal (colostrum) responses to this antigen. Furthermore piglets suckling from the vaccinated dams were protected against colonisation with *E. coli* O157. Interestingly a lower dose inoculum (10^6 cfu) was used in this study.

It is evident though that in most adult cattle there is no systemic immune response to Int280. In order for there to be anti-Int280 IgG in the colostrum a cow must have had recent exposure to Int280 that generated a systemic response. The lack of any adult immune response to Int280 would suggest that this is not the case.

Results described in chapter three show that R3 core LPS is common among the commensal *E. coli* of cattle. The association of the R3 core with *E. coli* O157 and other VTEC as observed by Currie and Poxton (1999) and Amor *et*

al (2000) means that this increased exposure to R3 is likely due in part to VTEC among the commensal flora. In a study looking at the prevalence of VTEC in healthy adult cattle, Blanco *et al* (1996) observed that 10% of the VTEC isolated from cattle were also positive for the *eae* gene. Orden *et al* (1998) noted similar findings albeit in diarrhoeic calves. It is therefore possible that a number of bovine commensals harbour the LEE pathogenicity island, and it is these organisms that provide the constant stimulus to the mucosal immune system. At birth the neonatal calf is fully immunocompetent but immunologically naïve and is exposed to a wide range of potential pathogens very quickly (Barington & Parish, 2001). The colostrum provides protection while the calf begins to produce its own antibody. It is therefore very likely that LEE positive organisms in the mother's commensal flora provide the Int280 and EspB challenge that leads to the immune response we detect in these calves. The porous nature of the gut in this period of the calf's life would lend itself well to the passage of antigens into the blood resulting in the generation of a systemic IgG response. As the calf matures the level of protection offered by the local mucosal immune system increases, and the barrier to colonisation provided by the commensal flora improves. If the innate and acquired facets of the mucosal immune system function well then it is possible that a subsequent re-challenge to the systemic immune system by a LEE positive organism is less likely. This would explain the low level of antibody to Int280 among adult cattle, as any systemic response would be short-lived and possibly undetectable after a year without constant re-challenge. Colostrum however would contain anti-

Int280 antibodies as the mucosal immune system will be constantly exposed to Int280 and other LEE encoded antigens.

The level of IgA to EspB and Int280 was examined using faecal samples treated with protease inhibitors to preserve the immunoglobulin. Initially dot blots were used to detect anti-Int280 or EspB IgA. Detectable levels of IgA were found in only a few of the samples investigated. Despite attempts to process the samples as soon as possible after collection, delays meant that the exact time between collection and processing was often very variable. It is likely that protease destruction of IgA prevented detection in many of the faecal samples. The time taken for faeces to pass through the digestive tract of a calf is considerable and therefore a degree of protease activity will already have occurred. Detection could have been improved by starting with a larger sample of faeces. Filter sterilisation would also have considerably reduced the protein content of the samples since the membranes used in these filters are nitrocellulose. Low protein binding filters were unavailable. ELISA proved a better technique for IgA detection but levels in all the animals were very low. The level of IgA over the four-week period of experimental infection did not change in any of the animals.

The LPS of *E. coli* O157 was the only antigen found to elicit an immune response. Four experimentally challenged calves negative for anti-*E. coli* O157 LPS IgG prior to challenge were subsequently found to have mounted a response against the LPS. Chart *et al* (2002) recently reported the

detection of anti-*E. coli* O157 LPS antibodies in a pregnant woman with haemolytic uraemic syndrome. Two days after the onset of diarrhoea, anti-O157 LPS IgM was detected in the sera of the patient. By day ten this response had diminished and an IgG response began to develop. It is clear that *E. coli* O157 LPS does induce an immune response, although in this case the development of disease will have facilitated the production of antibody. However Currie *et al* (2001) noted that a number of healthy adults used in their study had anti-O157 LPS IgG, an indication that immune responses to *E. coli* O157 LPS may be acquired in health. Currie *et al* (2001) showed that the response to the LPS of *E. coli* O157 in health could be largely attributed to the R3 core and it is unclear whether or not the responses detected in the experimentally infected calves or in the study by Chart *et al* (2002) are core specific or due in part to the O-antigen.

No response was detected in four calves (c87, c108, c117 and c121) and in two calves (c106 and c140) the response detected was low and did not change. The results observed in calves c106 and c117 are likely due to the fact that neither animal was colonised by the *E. coli* O157 inoculum. On the whole there was no correlation between the ability of the inoculum to colonise and the level of antibody reactive to O157 LPS. Although no *E. coli* O157 was detected in the faeces of calf c81 at any point during the experiment, it elicited the best response to LPS of all the calves. It would be interesting to determine at what point during the experimental infection the LPS response developed. If the IgG response was rapid and developed within three days

(prior to the first faecal sample) it may have helped prevent colonisation of the inoculum in this animal. Other animals that shed consistently well throughout the experiment, for example c95 showed a very muted response to the LPS.

A study was undertaken to ascertain whether or not the antibodies detected in the sera of the experimentally infected calves function to prevent AE lesion formation. Fernandes *et al* (2001) showed that IgA obtained from human colostrum inhibited enteroaggregative *E. coli* from binding to HEp-2 cells by binding and neutralising the aggregative adherence fimbrial adhesin II. Dean-Nystrom *et al* (2002) found that colostrum from dams vaccinated with purified Intimin contained antibodies reactive to the C-terminal region of Intimin responsible for receptor binding. Pre-treatment of *E. coli* O157 with colostrum from the vaccinated dams was found to significantly reduce the ability of the bacteria to form AE lesions on a HEp-2 monolayer.

Without bovine serum *E. coli* O157 Zap193 was shown to produce many AE lesions. The addition of bovine serum had no apparent affect on the ability of the *E. coli* O157 to cause AE lesions. In many cases the bovine serum appeared to 'improve' the quality and quantity of the lesions observed.

In order to determine if the bovine serum had had any effect upon AE lesion formation, a method of quantification had to be devised. It was decided that 100 cell nuclei and the number of lesions associated with these cells would

be counted. Owing to slow image reproduction and the rapidity with which the fluorescent image diminished, it was almost impossible to count the lesions. It was also noted that there was an uneven distribution of AE lesions across the HeLa cell monolayers. While areas of total confluence were devoid of lesions, many could be seen in areas where the HeLa cells were only partially confluent. This could be due to a preference *E. coli* O157 Zap 193 has for binding to the edge as opposed to the upper surface of a cell. Any reason for this is unclear.

Far from inhibiting the formation of AE lesions, the addition of bovine serum from experimentally challenged calves appeared to increase the size of micro-colonies and the number of the AE lesions observed. This is possibly due to antibody-mediated agglutination of the bacteria. *In vivo* opsonisation and agglutination of bacteria by antibody facilitates uptake by macrophages and phagocytes. On a HeLa cell monolayer however, agglutination of the bacteria is not followed by phagocytosis and the bacteria are left to sit on the HeLa cell. Wolff *et al* (1998) noted that contact with HeLa cells activated the type III secretion system, it is possible therefore that antibody-mediated agglutination facilitates contact between large numbers *E. coli* O157 and the HeLa cells and increases the number of AE lesions observed.

Conclusions

Since 1982 *E. coli* O157 has emerged as a serious human pathogen. Cattle are known to be the reservoir for these bacteria and *E. coli* O157 disease in humans is most often associated with direct contact with animal faeces or indirectly through the consumption of contaminated food. While adult cattle appear unaffected by *E. coli* O157 and able to transiently carry and shed (Griffin and Tauxe, 1991), mild disease in very young calves has been noted (Brown *et al*, 1997). A number of studies have been able to reproduce EHEC pathology (microvillus effacement and AE lesion formation) in bovine tissue explants, colostrum deprived and neonatal calves. Thus the answer to the question of whether or not cattle are intrinsically resistant to *E. coli* O157, is no. Indeed a close relative of *E. coli* O157, *E. coli* O26, is a well-recognised bovine and human pathogen, causing similar disease in both (Gunning *et al*, 2001).

The observation that colostrum deprived, or very young, immunologically naïve calves are susceptible to *E. coli* O157 suggests an important role for the bovine immune system in protection against EHEC disease. The study outlined in this thesis combined cloning technology with immunological and molecular detection techniques to examine the role of the bovine gastrointestinal microbiota and immune response in healthy and experimentally challenged cattle in the protection against *E. coli* O157 associated disease.

A four-week period of experimental *E. coli* O157 infection in 22 conventionally reared male calves failed to stimulate an immune response to either Int280 or EspB. Many of the calves had antibodies reactive to Int280 prior to challenge and the period of experimental infection did not alter the level of the response detected. In those animals naïve to Int280 prior to challenge, no response was detected at any point during the experimental infection. In general there appeared to be less detectable antibody to EspB than to Int280, although no direct comparisons may be made. In most cases levels of anti-EspB antibody before, and after challenge did not change.

A study was carried out to determine the distribution of the five core types of *E. coli* within two populations of faecal *E. coli* isolates from healthy humans and cattle. In accordance with the findings of other studies, R1 core LPS was the most frequently isolated core type in both populations (Amor *et al*, 2000, Gibb *et al*, 1992). The levels of R2 and R4 core LPS were similar within both groups of isolates, with R4 making up slightly more of the faecal bovine isolates. The level of R3 core detected among the bovine isolates was significantly higher than the level detected among the human isolates. Studies by Currie and Poxton (1999) and Amor *et al* (2000) demonstrating an association between R3 core LPS and VTEC suggest that the significantly higher level of R3 core LPS among bovine faecal *E. coli* isolates could be due to VTEC present in the commensal flora of cattle. Furthermore work by Blanco *et al* (1996) showing that a number of bovine VTEC isolates carry the LEE pathogenicity island, suggests that the elevated level of R3 exposure

among cattle could be due in part to EHEC in the bovine commensal flora. This is important when considering the results presented in chapter six which showed that most adult cattle examined had no detectable antibody to Int280. This is in contrast to the often-high levels of anti-Int280 antibody detected in the sera of a number of the calves prior to experimental challenge. Although all calves were screened for *E. coli* O157 carriage prior to experimental infection, the production of LEE antigens by other *E. coli* not detected may have induced the immune response detected in many of the animals.

Although a number of studies have shown that bovine, human and porcine colostra (Dean-Nystrom *et al* 2002, Lissner *et al*, 1996, Parissi-Crivelli *et al*, 2000) contains antibodies reactive to EHEC/EPEC virulence determinants, for there to be IgG reactive to Int280 in bovine colostra, there must be constant exposure to this antigen so as to generate memory cells able to produce antibody. The lack of a systemic response to Int280 among adult cattle suggests that the incidence of systemic exposure to antigens such as Int280 is low and that bovine colostra might not contain anti-Int280 IgG.

One possible explanation for these observations is that at birth calves encounter a large number of organisms very quickly and will generate antibody responses against most of the antigens they encounter (Barrington and Parish, 2001). As mentioned previously it is possible that a number of these organisms contain the LEE pathogenicity island and hence have the

capacity to produce Intimin and EspB. Thus in experimentally infected calves, antibodies detected prior to challenge could have been generated at or soon after birth upon exposure to the mother's commensal flora. As the mucosal immunity in these animals develops, systemic challenges may begin to fall off resulting in the decline of the IgG response detected. This would explain why adult cattle appear to have little or no antibody reactive to Intimin or EspB. The constant challenge to the gastrointestinal mucosa will perpetuate the mucosal immune response and could lead to the production of IgA reactive to LPS, Intimin and EspB in colostrum.

Work by Hoque *et* al (2000) linked increased exposure to diarrhoeagenic enterobacterial species with the development of an anti-core LPS antibody response that was possibly protective. An investigation of healthy human and cattle sera showed that in cattle the level of anti-R3 core antibody was significantly higher than the response to R1, R2 and K12. In humans no significant differences between the levels of anti-R1, R2 and R3 core LPS were observed. Currie *et al* (2000) demonstrated that mucosal immunity to LPS may play an important role in protection from EHEC disease. It is possible therefore that the increased exposure to R3 core LPS in cattle leads to a protective immune response, that provides an effective mucosal protection. Data presented in this thesis show that LPS was the only antigen found to stimulate an immune response in any of the experimentally infected calves. While the role of this immune response is unclear it may play an

important part in the prevention of EHEC colonisation. Further work is required to fully characterise this response.

No anti-EspB or Int280 IgA was detected in the faecal antibody preparations of any of the calves tested. The level of mucosal immunity needs to be studied and future work should take into consideration the rapid degradation of IgA in faecal samples. Protease inhibitors should be added to freshly collected faeces as soon as possible to minimise IgA loss. The use of protease inhibitor tablets may make this easier.

While recombinant antigens offer an opportunity to examine the immune response to individual components of a virulence network such as the LEE pathogenicity island, it should be noted that due to the large GST tag the antigenicity of the protein could have been altered. The EspB protein was generated with a number of errors and the low responses detected in the experimentally infected calves could have resulted from the defects destroying vital epitopes. Any future work would be recommended to use a smaller protein tag, for example the *His*-tag system, which might have less of an impact on the recombinant conformation.

Owing to the limitations of time it was possible to generate only Int280 from EDL933 (Int280γ). It is possible that other intimin types show varying degrees of immunogenicity in cattle and could provide an answer as to why *E. coli* O26 causes bovine disease. It would be interesting to conduct a number of experimental infections with other *E. coli* strains expressing different intimin types.

The demonstration by Hoey *et al* (2002) that the receptor for verotoxin, Gb_3 is present on bovine intestinal tissue leads to the possibility that antibody-mediated protection may neutralise the effects of this toxin in cattle. The generation of a functional recombinant verotoxin B subunit would be a vital tool in helping to ascertain a role for any antibody reactive to the toxin. Although it was not possible to generate recombinant Vt2B here, a number of studies have achieved it through different means (Gunzer and Karch, 1993, Acheson and Breucher 1994, Byun *et al*, 2001). It would be worthwhile pursuing these ideas and utilising some of the technology to generate a recombinant B-subunit.

There is hope that an intimin-based vaccine may provide a way of indirectly avoiding human disease by preventing the colonisation of *E. coli* O157 in cattle. This thesis has shown that despite often-large levels of anti-Int280 or EspB IgG, the ability of *E. coli* O157 to colonise calves appears unaffected. The proteolytic Int280 and EspB fragments generated during the protein expression and purification procedures appeared to exhibit varying degrees of immunogenicity. This was particularly noticeable with the Int280 fragments. It would be interesting to examine which regions of Intimin are most immunogenic in cattle. The results of such studies may be able to pinpoint fragments of intimin that offer vaccine potential. It is possible that Intimin expression is under considerable regulation in the bovine host and that other factors for example pili are more important in initial adhesion. Thus vaccine development should not only look at the possibility of Intimin variants with differing immunogenicity but other factors that may offer better targets.

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Appendix 1

Buffers and solutions

Phosphate buffered saline (PBS)

Dulbecco A tablets (Oxoid BR14), One tablet in 100ml dH_2O

Polyacrylamide gel electrophoresis (Laemmli, 1970)

Double strength separating gel buffer (0.75M Tris-HCl, pH8.8, 0.2% w/v sodium lauryl (dodecyl) sulphate: SDS)

Tris (hydroxymethyl) methylamine	91g
SDS	2g
PfH_2O	1000ml

Double strength stacking buffer (0.25M Tris-HCl, pH6.8, 0.2% w/v SDS)

Tris (hydroxymethyl) methylamine	15g
SDS	1g
PfH_2O	500ml

SDS-PAGE Electrode buffer (0.025M Tris, 0.192M glycine, 0.1%w/v SDS, pH8.3)

Tris (hydroxymethyl) methylamine	6g
Glycine (chromatographically homogeneous)	29g
SDS	2g
PfH_2O	2000ml

Double strength sample buffer (0.125M Tris-HCl, pH6.8, 4% w/v SDS, 20% v/v glycerol, 2% v/v 2-mercaptoethanol, 0.002% v/v bromophenol blue)

Tris (hydroxymethyl) methylamine	1.5g
SDS	4g
Glycerol (20% v/v)	25g
2-mercaptoethanol	2ml
Bromophenol blue (0.05% aqueous solution)	4ml
PfH_2O	100ml

Immunoblotting

Western blotting electrode buffer (0.025M Tris, 0.192M Glycine, 20% v/v methanol, pH8.3)

Tris (hydroxymethyl) methylamine	6g
Glycine	29g
Methanol	400ml
dH_2O	1600ml

Tris buffered saline (TBS: 0.02M Tris-HCl, 0.5M NaCl, 0.25% v/v, pH7.5)

Tris (hydroxymethyl) methylamine	4.8g
NaCl	58.5g
dH_2O	2000ml

Tween Tris buffered saline (TTBS: 0.02M Tris-HCl, 0.5M NaCl, Tween-20pH7.5)

Tris (hydroxymethyl) methylamine	4.8
NaCl	58.5
Tween 20	0.5ml
dH_2O	2000ml

Blocking solution

3% fish gelatin (v/v) in TBS

Antibody buffer

1% fish gelatin (v/v) in TBS

Horseradish peroxidase (HRP) colour development solution A

4-chloro-1-naphthol	60mg
Methanol	20ml

Horseradish peroxidase (HRP) colour development solution B

Hydrogen peroxide	60µl
TBS	100ml

Solutions A and B were mixed immediately prior to use

Coomassie Blue stain (Hancock and Poxton, 1988)

SDS-PAGE proten gels were incubated over night in solution one and destained the following day in solutions 2-4 (1h in each stain)

	Coomassie brilliant blue R-250	Propan-2-ol	Acetic acid (Glacial)	Methanol	dH_2O
Solution 1	1000mg	500ml	200	-	1300ml
Solution 2	100mg	200ml	200	-	1600ml
Solution 3	48mg	-	200	-	1800ml
Solution 4	-	-	200	800	1000ml
Solution 5	-	-	200	-	1800ml

ıents for pCR®4-TOPO
ıucleotides

Invitrogen™
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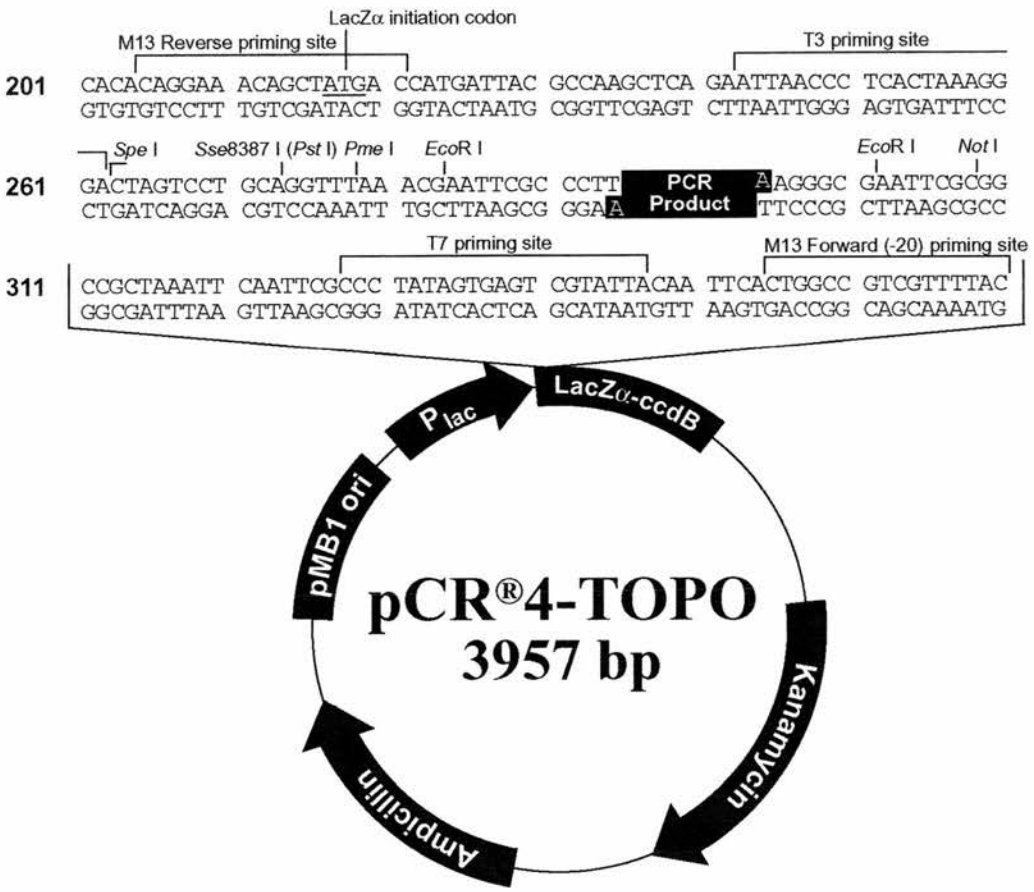
ɔmoter region: bases 2-216
CAP binding site: bases 95-132
RNA polymerase binding site: bases 133-178
Lac repressor binding site: bases 179-199
f transcription: base 179
ɛeverse priming site: bases 205-221
-*ccdB* gene fusion: bases 217-810
LacZα portion of fusion: bases 217-497
ccdB portion of fusion: bases 508-810
ming site: bases 243-262
ker: bases 262-312
™ Cloning site: bases 294-295
ming site: bases 328-347
orward (-20) priming site: bases 355-370
neo (kanamycin) promoter region: bases 1021-1070
neo (kanamycin) resistance gene (ORF): bases 1159-1953
bla promoter region: bases 2062-2156
RNA polymerase binding site: bases 2062-2143
-35 region: bases 2087-2093
bla promoter (P3): bases 2116-2122
Start of transcription: base 2122
Ribosome binding site: bases 2145-2149
bla (ampicillin) resistance gene (ORF): bases 2157-3017
pMB1 origin (pUC-derived): bases 3162-3835

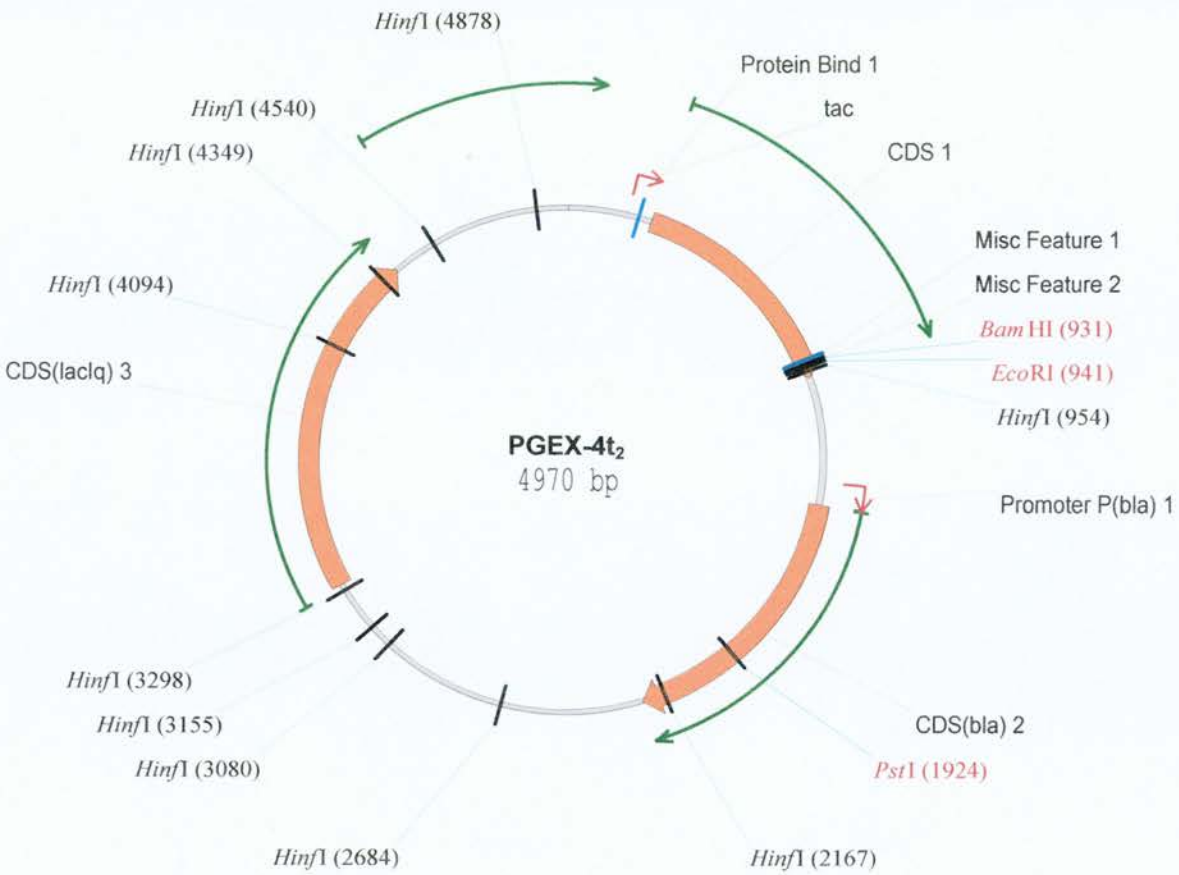


ɜquence of pCR®4-TOPO has been compiled from information in sequence databases, published
ıces, and other sources. This vector has not yet been completely sequenced. If you suspect an error in
ɜuence, please contact Invitrogen's Technical Service Department.

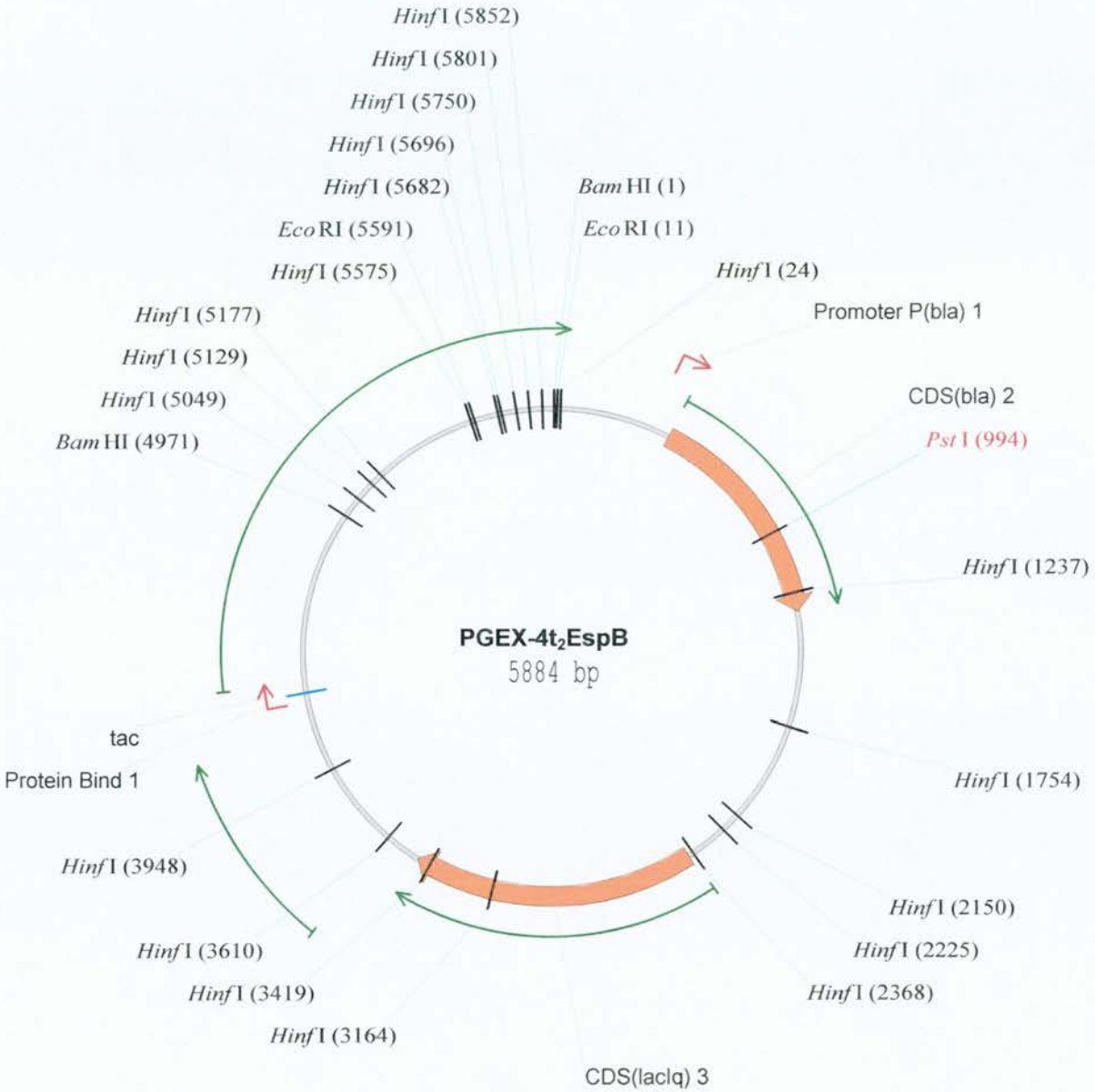
Appendix 2

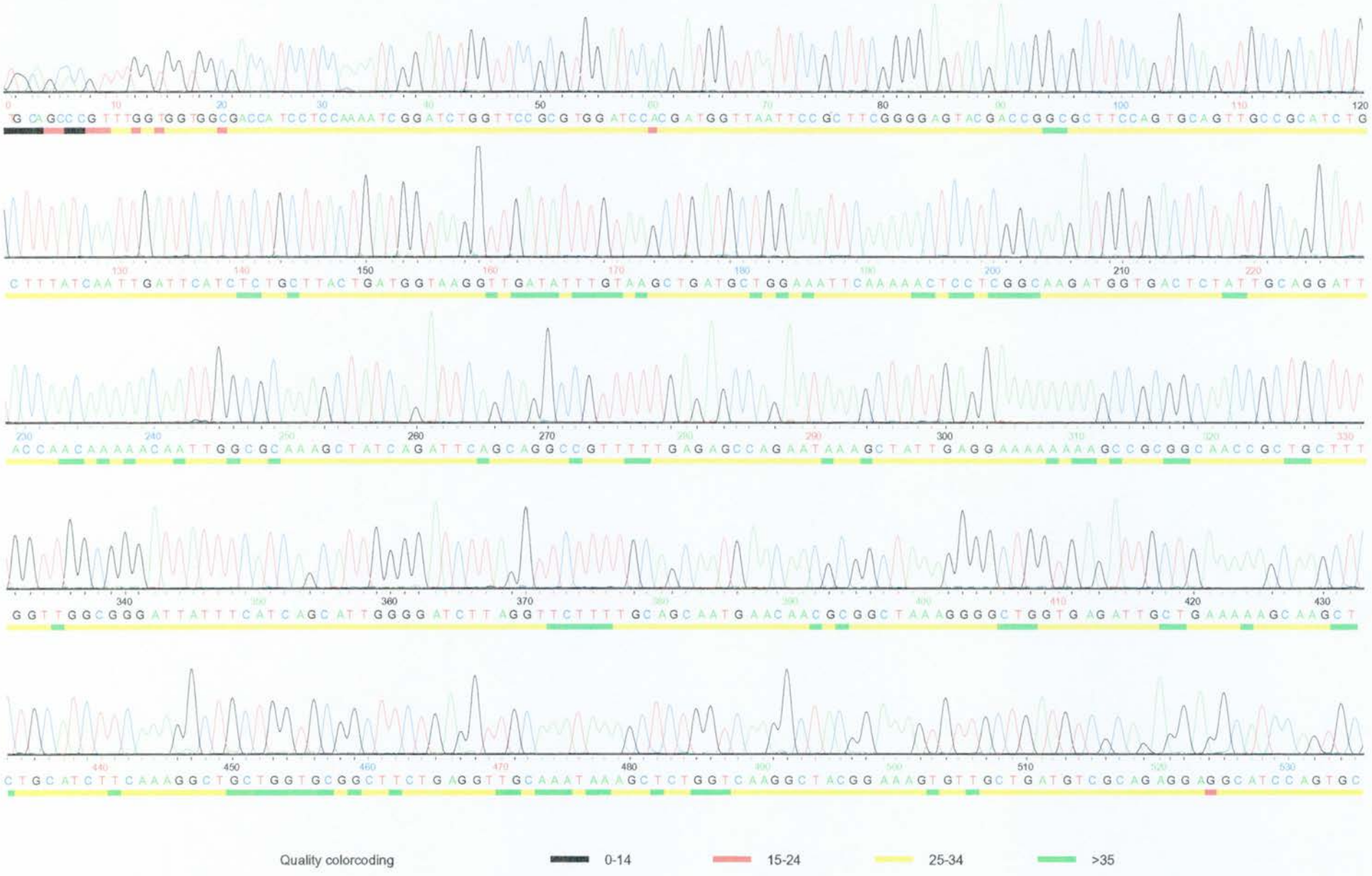
Vector maps and sequence information

pGEX4t$_2$ – commercial expression vector (Amersham Pharmacia)



pGEX-4t$_2$ + EspB

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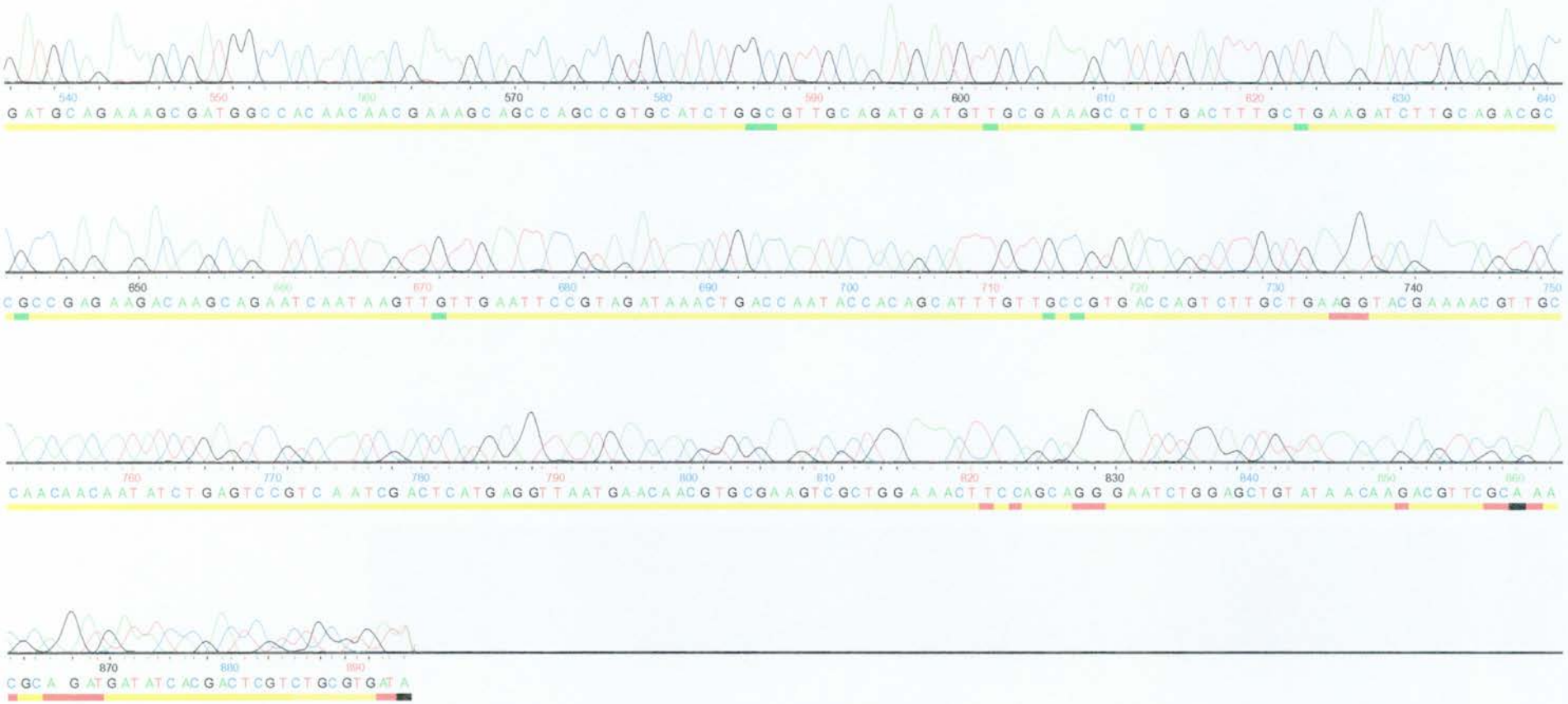

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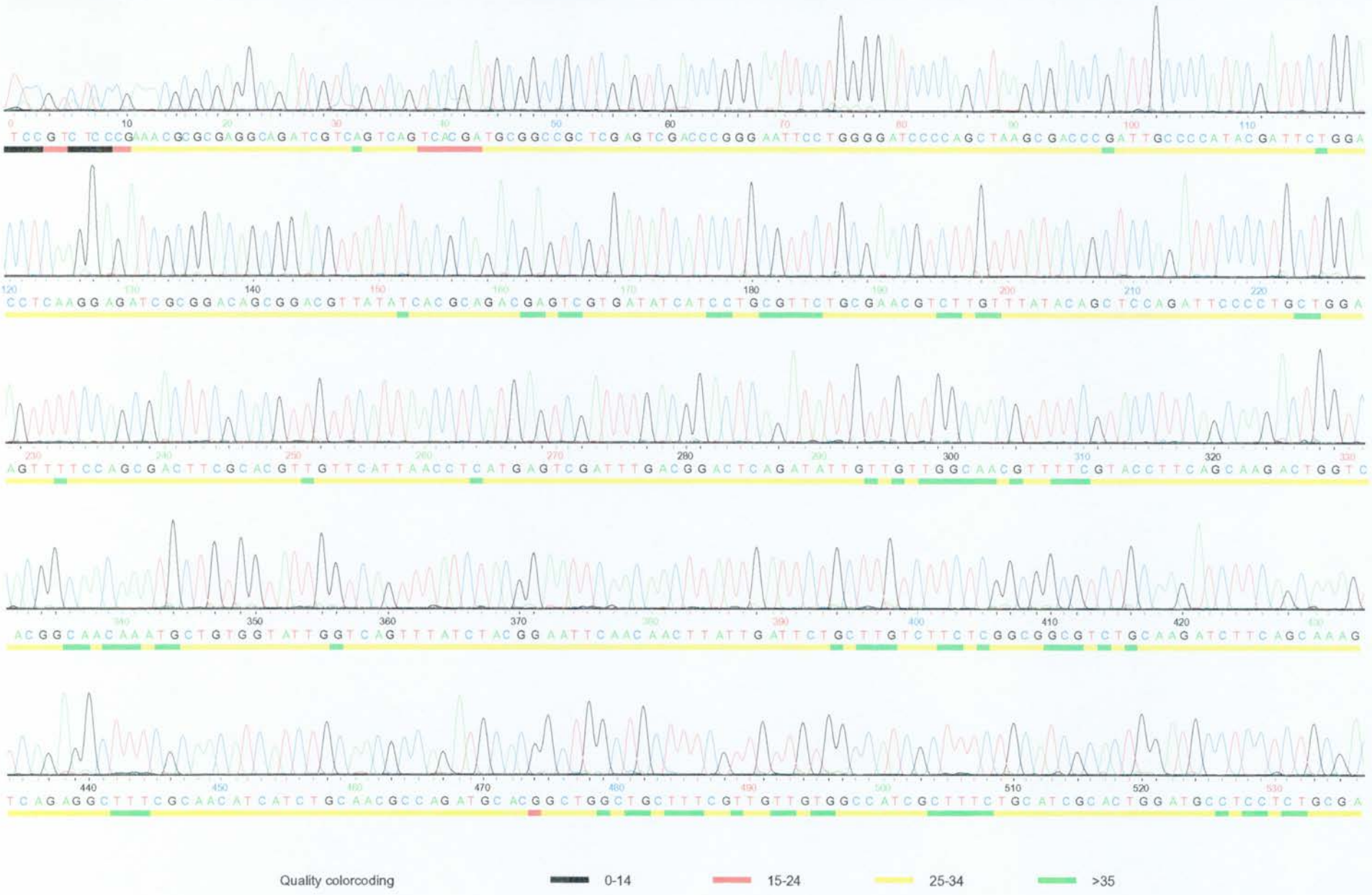
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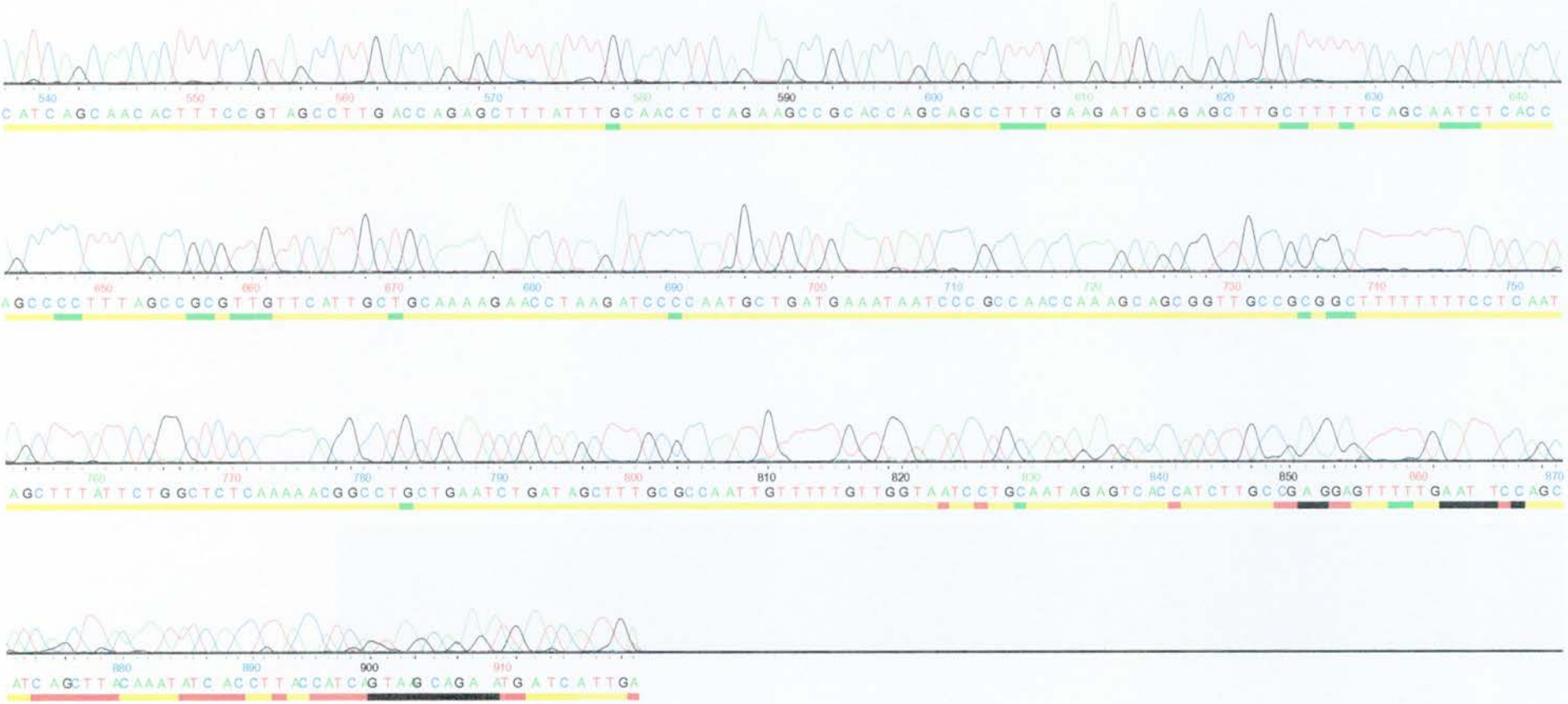
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760 770 780 790 800 810 820 830 840 850 860 870
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Quality colorcoding
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15-24
25-34
>35

ClustalW Multiple Sequence Alignment Results : EspB : pGEX for/rev sequences

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2 SampleEspBf-pGexfor   --------------- ------------ACG ATGGTTAATTCCGCT TCGGGGAGTACGACC GGCGCTTCCAGTGCA GTTGCCGCATCTGCT      63
3 SampleEspBr-pGexrev   --------------- --------------- --------------- --------------- --------------- ---------------       0

                        91          105 106         120 121         135 136         150 151         165 166         180
1 EspB                  TTATCAATTGATTCA TCTCTGCTTACTGAT GGTAAGGTTGATATT TGTAAGCTGATGCTG GAAATTCAAAAACTC CTCGGCAAGATGGTG     180
2 SampleEspBf-pGexfor   TTATCAATTGATTCA TCTCTGCTTACTGAT GGTAAGGTTGATATT TGTAAGCTGATGCTG GAAATTCAAAAACTC CTCGGCAAGATGGTG     153
3 SampleEspBr-pGexrev   ---TCAAT-GAT-CA T-TCTGCTTACTGAT GGTAAGGT-GATATT TGTAAGCTGATGCTG GAA-TTCAAAAACTC CTCGGCAAGATGGTG      82

                        181         195 196         210 211         225 226         240 241         255 256         270
1 EspB                  ACTCTATTGCAGGAT TACCAACAAAAACAA TTGGCGCAAAGCTAT CAGATTCAGCAGGCC GTTTTTGAGAGCCAG AATAAAGCTATTGAG     270
2 SampleEspBf-pGexfor   ACTCTATTGCAGGAT TACCAACAAAAACAA TTGGCGCAAAGCTAT CAGATTCAGCAGGCC GTTTTTGAGAGCCAG AATAAAGCTATTGAG     243
3 SampleEspBr-pGexrev   ACTCTATTGCAGGAT TACCAACAAAAACAA TTGGCGCAAAGCTAT CAGATTCAGCAGGCC GTTTTTGAGAGCCAG AATAAAGCTATTGAG     172

                        271         285 286         300 301         315 316         330 331         345 346         360
1 EspB                  GAAAAAAAAGCCGCG GCAACCGCTGCTTTG GTTGGCGGGATTATT TCATCAGCATTGGGG ATCTTAGGTTCTTTT GCAGCAATGAACAAC     360
2 SampleEspBf-pGexfor   GAAAAAAAAGCCGCG GCAACCGCTGCTTTG GTTGGCGGGATTATT TCATCAGCATTGGGG ATCTTAGGTTCTTTT GCAGCAATGAACAAC     333
3 SampleEspBr-pGexrev   GAAAAAAAAGCCGCG GCAACCGCTGCTTTG GTTGGCGGGATTATT TCATCAGCATTGGGG ATCTTAGGTTCTTTT GCAGCAATGAACAAC     262

                        361         375 376         390 391         405 406         420 421         435 436         450
1 EspB                  GCGGCTAAAGGGGCT GGTGAGATTGCTGAA AAAGCAAGCTCTGCA TCTTCAAAGGCTGCT GGTGCGGCTTCTGAG GTTGCAAATAAAGCT     450
2 SampleEspBf-pGexfor   GCGGCTAAAGGGGCT GGTGAGATTGCTGAA AAAGCAAGCTCTGCA TCTTCAAAGGCTGCT GGTGCGGCTTCTGAG GTTGCAAATAAAGCT     423
3 SampleEspBr-pGexrev   GCGGCTAAAGGGGCT GGTGAGATTGCTGAA AAAGCAAGCTCTGCA TCTTCAAAGGCTGCT GGTGCGGCTTCTGAG GTTGCAAATAAAGCT     352

                        451         465 466         480 481         495 496         510 511         525 526         540
1 EspB                  CTGGTCAAGGCTACG GAAAGTGTTGCTGAT GTCGCAGAGGAGGCA TCCAGTGCGATGCAG AAAGCGATGGCCACA ACAACGAAAGCAGCC     540
2 SampleEspBf-pGexfor   CTGGTCAAGGCTACG GAAAGTGTTGCTGAT GTCGCAGAGGAGGCA TCCAGTGCGATGCAG AAAGCGATGGCCACA ACAACGAAAGCAGCC     513
3 SampleEspBr-pGexrev   CTGGTCAAGGCTACG GAAAGTGTTGCTGAT GTCGCAGAGGAGGCA TCCAGTGCGATGCAG AAAGCGATGGCCACA ACAACGAAAGCAGCC     442
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                     541         555 556         570 571         585 586         600 601         615 616         630
1 EspB               AGCCGTGCATCTGGC GTTGCAGATGATGTT GCGAAAGCCTCTGAC TTTGCTGAAGATCTT GCAGACGCCGCCGAG AAGACAAGCAGAATC     630
2 SampleEspBf-pGexfor AGCCGTGCATCTGGC GTTGCAGATGATGTT GCGAAAGCCTCTGAC TTTGCTGAAGATCTT GCAGACGCCGCCGAG AAGACAAGCAGAATC     603
3 SampleEspBr-pGexrev AGCCGTGCATCTGGC GTTGCAGATGATGTT GCGAAAGCCTCTGAC TTTGCTGAAGATCTT GCAGACGCCGCCGAG AAGACAAGCAGAATC     532

                     631         645 646         660 661         675 676         690 691         705 706         720
1 EspB               AATAAGTTGTTGAAT TCCGTAGATAAACTG ACCAATACCACAGCA TTTGTTGCCGTGACC AGTCTTGCTGAAGGT ACGAAAACGTTGCCA     720
2 SampleEspBf-pGexfor AATAAGTTGTTGAAT TCCGTAGATAAACTG ACCAATACCACAGCA TTTGTTGCCGTGACC AGTCTTGCTGAAGGT ACGAAAACGTTGCCA     693
3 SampleEspBr-pGexrev AATAAGTTGTTGAAT TCCGTAGATAAACTG ACCAATACCACAGCA TTTGTTGCCGTGACC AGTCTTGCTGAAGGT ACGAAAACGTTGCCA     622

                     721         735 736         750 751         765 766         780 781         795 796         810
1 EspB               ACAACAATATCTGAG TCCGTCAAATCGACT CATGAGGTTAATGAA CAACGTGCGAAGTCG CTGGAAAACTTCCAG CAGGGGAATCTGGAG     810
2 SampleEspBf-pGexfor ACAACAATATCTGAG TCCGTCAA-TCGACT CATGAGGTTAATGAA CAACGTGCGAAGTCG CTGGAAA-CTTCCAG CAGGG-AATCTGGAG     780
3 SampleEspBr-pGexrev ACAACAATATCTGAG TCCGTCAAATCGACT CATGAGGTTAATGAA CAACGTGCGAAGTCG CTGGAAAACTTCCAG CAGGGGAATCTGGAG     712

                     811         825 826         840 841         855 856         870 871         885 886         900
1 EspB               CTGTATAAACAAGAC GTTCGCAGAACGCAG GATGATATCACGACT CGTCTGCGTGATATA ACGTCCGCTGTCCGC GATCTCCTTGAGGTC     900
2 SampleEspBf-pGexfor CTGTATAA-CAAGAC GTTCGCA-AACGCAG -ATGATATCACGACT CGTCTGCGTGATA-- --------------- ---------------     835
3 SampleEspBr-pGexrev CTGTATAAACAAGAC GTTCGCAGAACGCAG GATGATATCACGACT CGTCTGCGTGATATA ACGTCCGCTGTCCGC GATCTCCTTGAGGTC     802

                     901         915 916         930 931         945 946         960 961         975 976         990
1 EspB               CAGAATCGTATGGGG CAATCGGGTCGCTTA GCTGGGTAA------ --------------- --------------- ---------------     939
2 SampleEspBf-pGexfor --------------- --------------- --------------- --------------- --------------- ---------------     835
3 SampleEspBr-pGexrev CAGAATCGTATGGGG CAATCGGGTCGCTTA GCTGGGGATCCCCAG GAATTCCCGGGTCGA CTCGAGCGGCCGCAT CGTGACTGACTGACG     892
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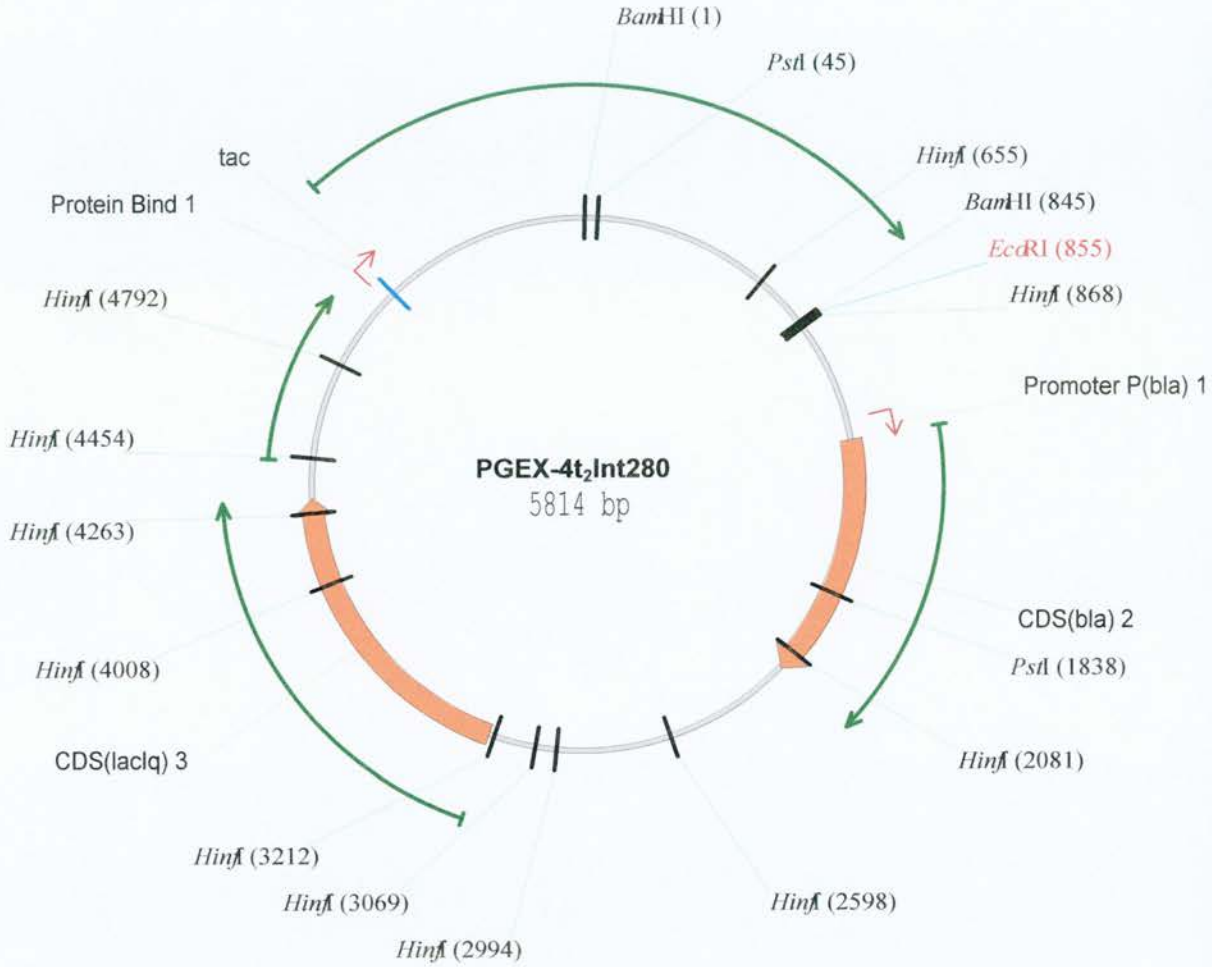
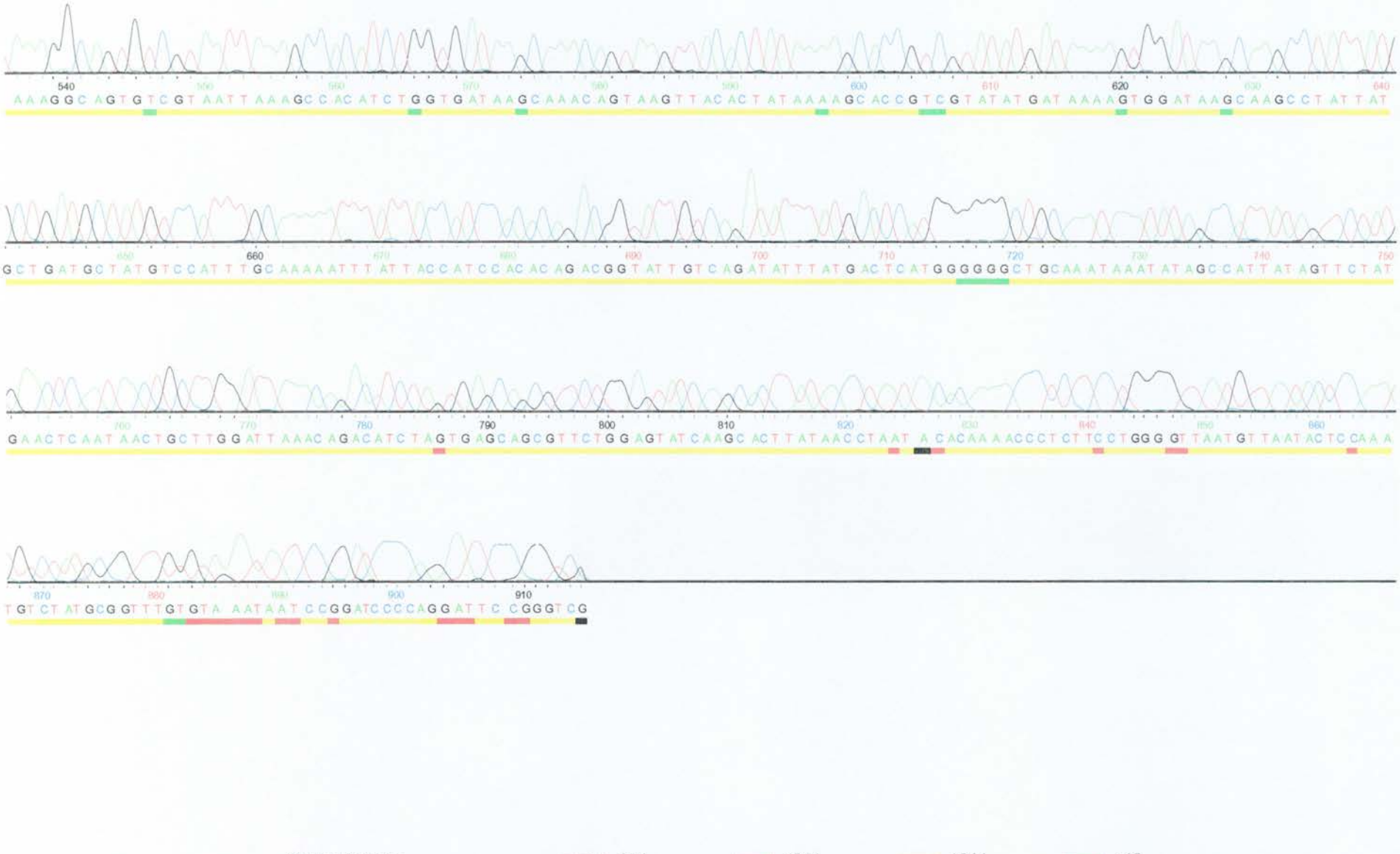
pGEX-4t$_2$ + Int280





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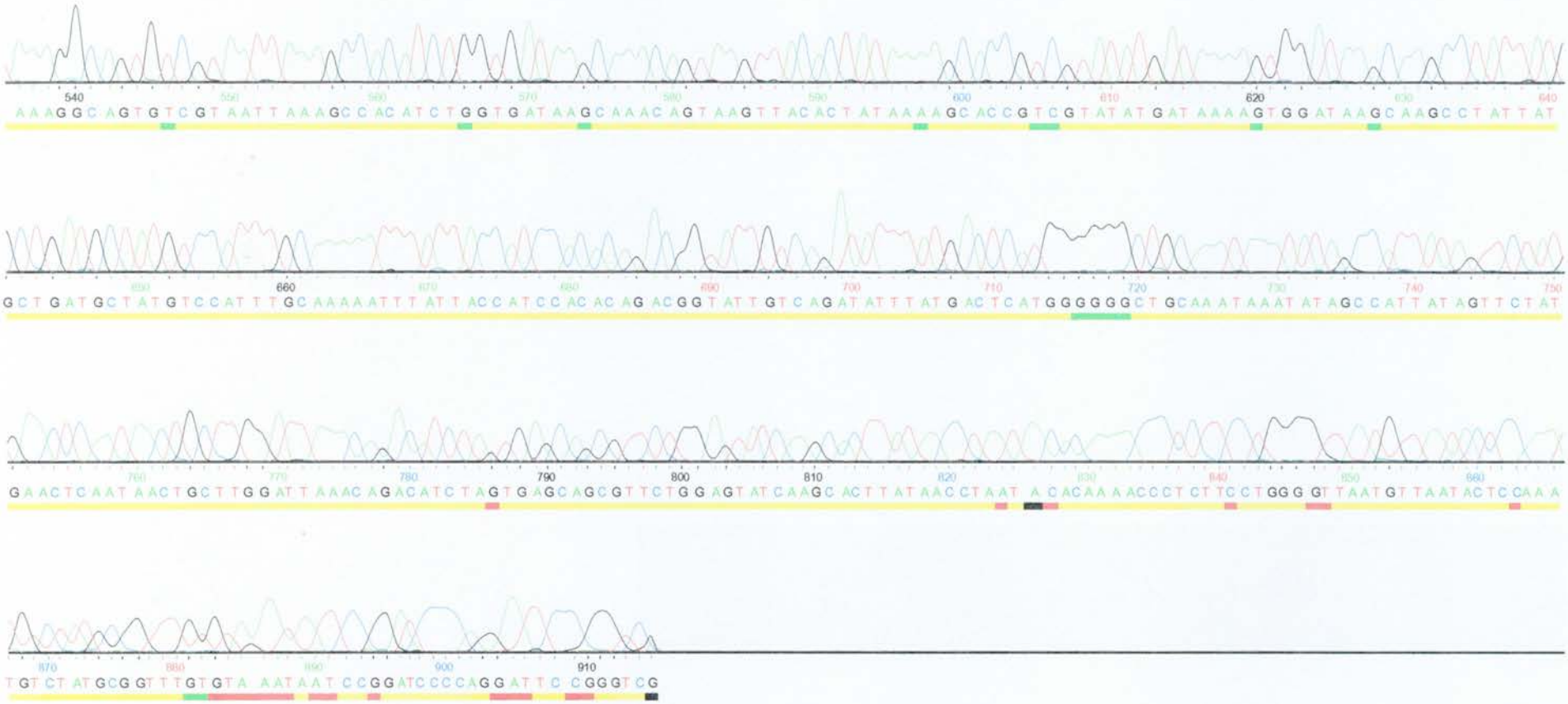

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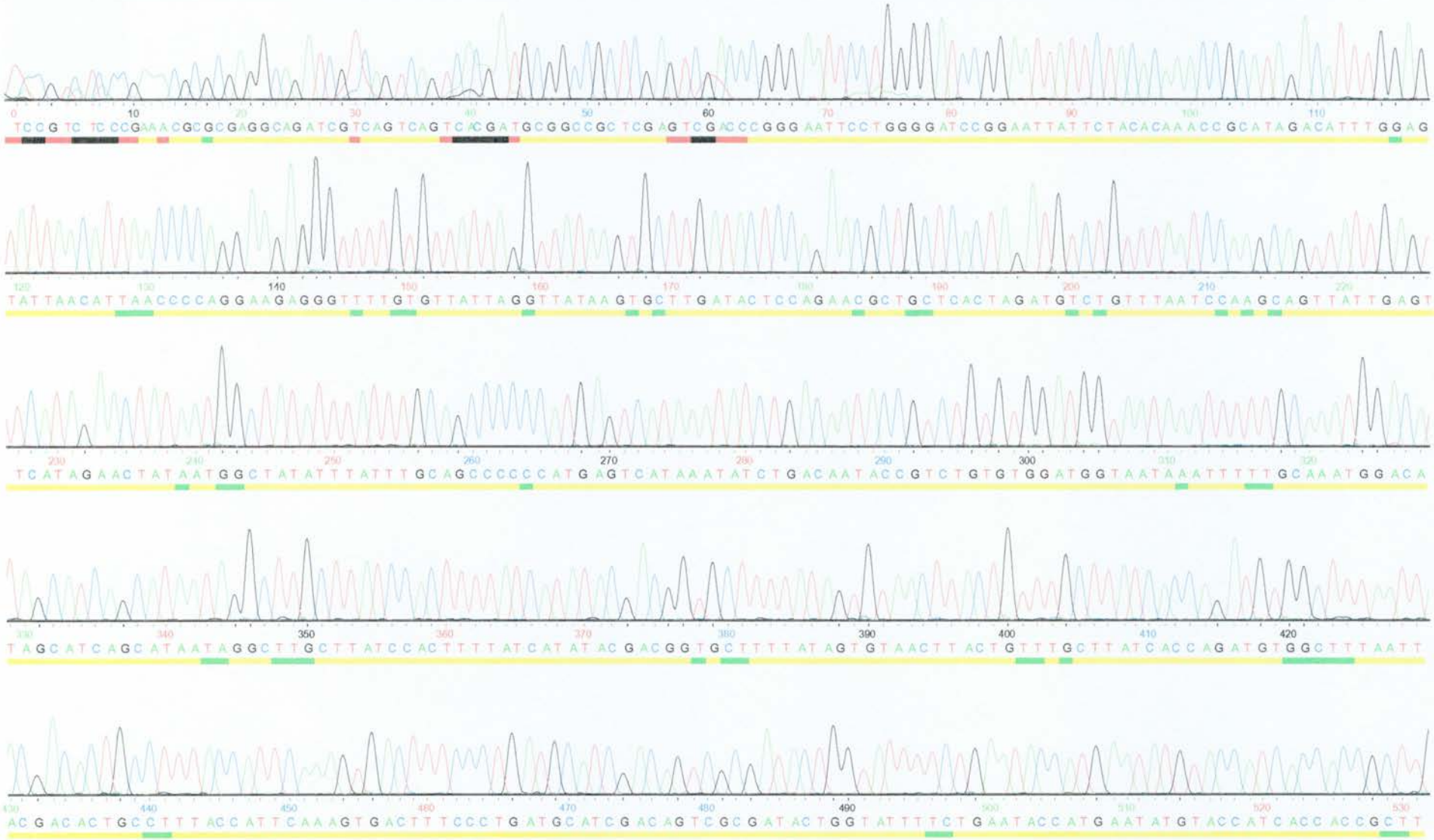
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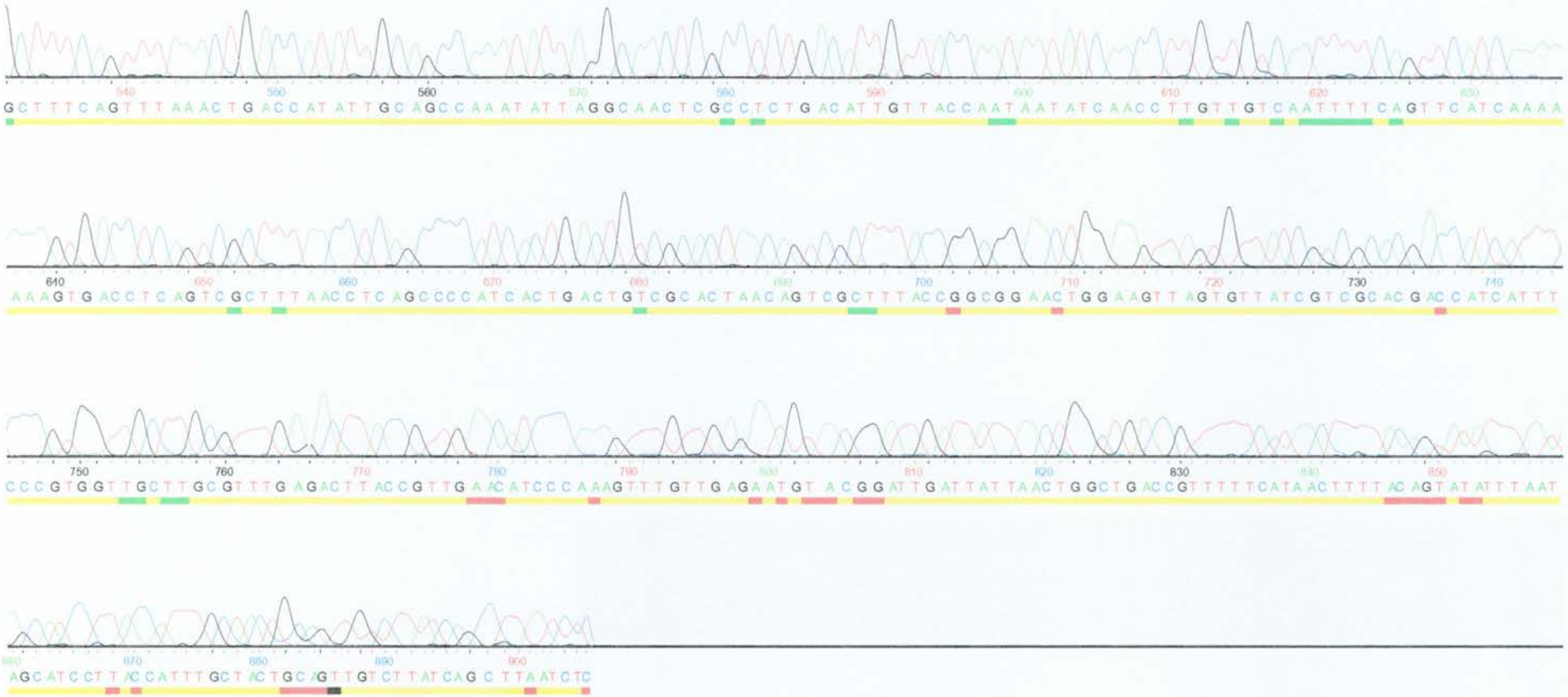

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ClustalW Multiple Sequence Alignment Results: Intimin γ : pGEX for/rev sequences

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1 Intimin               CAGGTCGTCGTGTCT GCTAAAACCGCGGAG ATGACTTCAGCACTT AATGCCAGTGCGGTT ATATTTTTTGATCAA ACCAAGGCCAGCATT   1980
2 SampleIntf-pGexfor    --------------- --------------- --------------- --------------- --------------- -------CCAGCATT      8
3 SampleIntr-pGexrev    --------------- --------------- --------------- --------------- --------------- ---------------      0

                        1981       1995 1996       2010 2011       2025 2026       2040 2041       2055 2056       2070
1 Intimin               ACTGAGATTAAGGCT GATAAGACAACTGCA GTAGCAAATGGTAAG GATGCTATTAAATAT ACTGTAAAAGTTATG AAAAACGGTCAGCCA   2070
2 SampleIntf-pGexfor    ACTGAGATTAAGGCT GATAAGACAACTGCA GTAGCAAATGGTAAG GATGCTATTAAATAT ACTGTACAAGTTATG AAAAACGGTCAGCCA     98
3 SampleIntr-pGexrev    ---GAGATTAAG-CT GATAAGACAACTGCA GTAGCAAATGGTAAG GATGCTATTAAATAT ACTGTAAAAGTTATG AAAAACGGTCAGCCA     86

                        2071       2085 2086       2100 2101       2115 2116       2130 2131       2145 2146       2160
1 Intimin               GTTAATAATCAATCC GTTACATTCTCAACA AACTTTGGGATGTTC AACGGTAAGTCTCAA ACGCAAGCAACCACG GGAAATGATGGTCGT   2160
2 SampleIntf-pGexfor    GTTAATAATCAATCC GTTACATTCTCAACA AACTTTGGGATGTTC AACGGTAAGTCTCAA ACGCAAGCAACCACG GGAAATGATGGTCGT    188
3 SampleIntr-pGexrev    GTTAATAATCAATCC GT-ACATTCTCAACA AACTTTGGGATGTTC AACGGTAAGTCTCAA ACGCAAGCAACCACG GGAAATGATGGTCGT    175

                        2161       2175 2176       2190 2191       2205 2206       2220 2221       2235 2236       2250
1 Intimin               GCGACGATAACACTA ACTTCCAGTTCCGCC GGTAAAGCGACTGTT AGTGCGACAGTCAGT GATGGGGCTGAGGTT AAAGCGACTGAGGTC   2250
2 SampleIntf-pGexfor    GCGACGATAACACTA ACTTCCAGTTCCGCC GGTAAAGCGACTGTT AGTGCGACAGTCAGT GATGGGGCTGAGGTT AAAGCGACTGAGGTC    278
3 SampleIntr-pGexrev    GCGACGATAACACTA ACTTCCAGTTCCGCC GGTAAAGCGACTGTT AGTGCGACAGTCAGT GATGGGGCTGAGGTT AAAGCGACTGAGGTC    265

                        2251       2265 2266       2280 2281       2295 2296       2310 2311       2325 2326       2340
1 Intimin               ACTTTTTTTGATGAA CTGAAAATTGACAAC AAGGTTGATATTATT GGTAACAATGTCAGA GGCGAGTTGCCTAAT ATTTGGCTGCAATAT   2340
2 SampleIntf-pGexfor    ACTTTTTTTGATGAA CTGAAAATTGACAAC AAGGTTGATATTATT GGTAACAATGTCAGA GGCGAGTTGCCTAAT ATTTGGCTGCAATAT    368
3 SampleIntr-pGexrev    ACTTTTTTTGATGAA CTGAAAATTGACAAC AAGGTTGATATTATT GGTAACAATGTCAGA GGCGAGTTGCCTAAT ATTTGGCTGCAATAT    355

                        2341       2355 2356       2370 2371       2385 2386       2400 2401       2415 2416       2430
1 Intimin               GGTCAGTTTAAACTG AAAGCAAGCGGTGGT GATGGTACATATTCA TGGTATTCAGAAAAT ACCAGTATCGCGACT GTCGATGCATCAGGG   2430
2 SampleIntf-pGexfor    GGTCAGTTTAAACTG AAAGCAAGCGGTGGT GATGGTACATATTCA TGGTATTCAGAAAAT ACCAGTATCGCGACT GTCGATGCATCAGGG    458
3 SampleIntr-pGexrev    GGTCAGTTTAAACTG AAAGCAAGCGGTGGT GATGGTACATATTCA TGGTATTCAGAAAAT ACCAGTATCGCGACT GTCGATGCATCAGGG    445
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                2431       2445 2446       2460 2461       2475 2476       2490 2491       2505 2506       2520
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2 SampleIntf-pGexfor AAAGTCACTTTGAAT GGTAAAGGCAGTGTC GTAATTAAAGCCACA TCTGGTGATAAGCAA ACAGTAAGTTACACT ATAAAAGCACCGTCG    548
3 SampleIntr-pGexrev AAAGTCACTTTGAAT GGTAAAGGCAGTGTC GTAATTAAAGCCACA TCTGGTGATAAGCAA ACAGTAAGTTACACT ATAAAAGCACCGTCG    535

                    2521       2535 2536       2550 2551       2565 2566       2580 2581       2595 2596       2610
1 Intimin            TATATGATAAAAGTG GATAAGCAAGCCTAT TATGCTGATGCTATG TCCATTTGCAAAAAT TTATTACCATCCACA CAGACGGTATTGTCA   2610
2 SampleIntf-pGexfor TATATGATAAAAGTG GATAAGCAAGCCTAT TATGCTGATGCTATG TCCATTTGCAAAAAT TTATTACCATCCACA CAGACGGTATTGTCA    638
3 SampleIntr-pGexrev TATATGATAAAAGTG GATAAGCAAGCCTAT TATGCTGATGCTATG TCCATTTGCAAAAAT TTATTACCATCCACA CAGACGGTATTGTCA    625

                    2611       2625 2626       2640 2641       2655 2656       2670 2671       2685 2686       2700
1 Intimin            GATATTTATGACTCA TGGGGGGCTGCAAAT AAATATAGCCATTAT AGTTCTATGAACTCA ATAACTGCTTGGATT AAACAGACATCTAGT   2700
2 SampleIntf-pGexfor GATATTTATGACTCA TGGGGGGCTGCAAAT AAATATAGCCATTAT AGTTCTATGAACTCA ATAACTGCTTGGATT AAACAGACATCTAGT    728
3 SampleIntr-pGexrev GATATTTATGACTCA TGGGGGGCTGCAAAT AAATATAGCCATTAT AGTTCTATGAACTCA ATAACTGCTTGGATT AAACAGACATCTAGT    715

                    2701       2715 2716       2730 2731       2745 2746       2760 2761       2775 2776       2790
1 Intimin            GAGCAGCGTTCTGGA GTATCAAGCACTTAT AACCTAATAACACAA AACCCTCTTCCTGGG GTTAATGTTAATACT CCAAATGTCTATGCG   2790
2 SampleIntf-pGexfor GAGCAGCGTTCTGGA GTATCAAGCACTTAT AACCTAATA-CACAA AACCCTCTTCCTGGG GTTAATGTTAATACT CCAAATGTCTATGCG    817
3 SampleIntr-pGexrev GAGCAGCGTTCTGGA GTATCAAGCACTTAT AACCTAATAACACAA AACCCTCTTCCTGGG GTTAATGTTAATACT CCAAATGTCTATGCG    805

                    2791       2805 2806       2820 2821       2835 2836       2850 2851       2865 2866       2880
1 Intimin            GTTTGTGTAGAATAA --------------- --------------- --------------- --------------- ---------------   2805
2 SampleIntf-pGexfor GTTTGTGTA-AATAA T-CCGGATCCCCAGG ATT--CCGGGTCG-- --------------- --------------- ---------------    856
3 SampleIntr-pGexrev GTTTGTGTAGAATAA TTCCGGATCCCCAGG AATTCCCGGGTCGAC TCGAGCGGCCGCATC GTGACTGACTGACGA TCTGCCTCGCGCGTT    895
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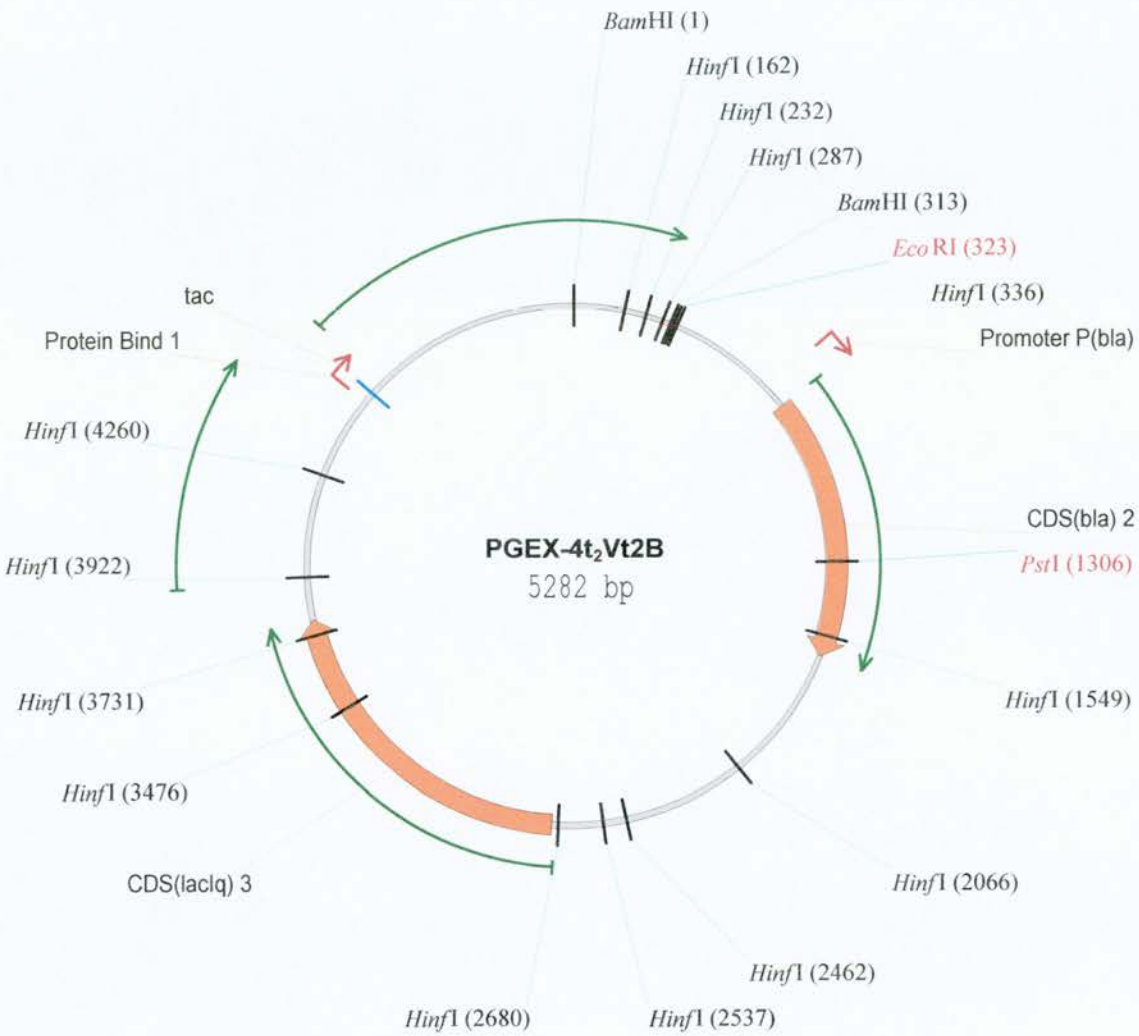
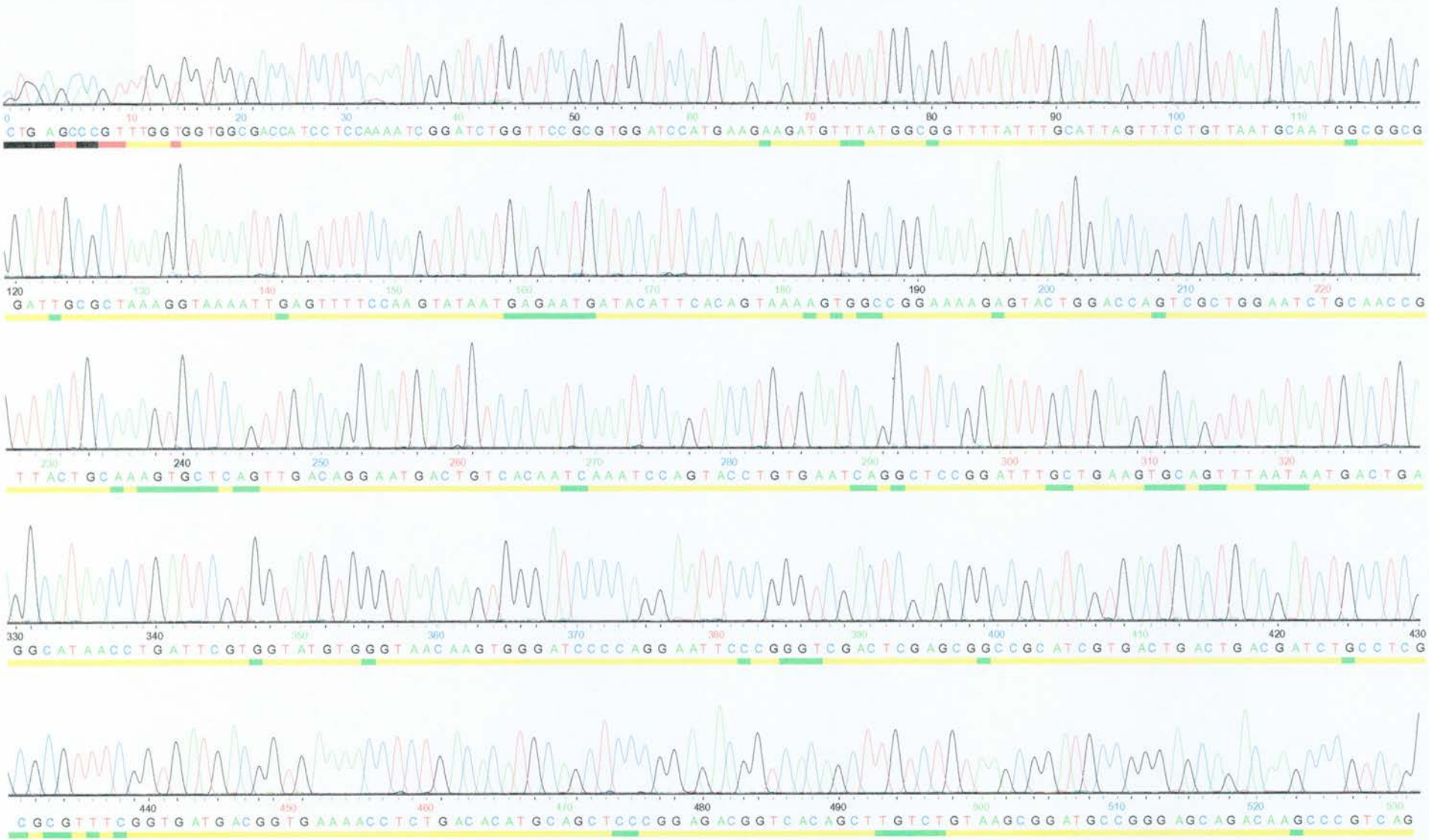
pGEX-4t$_2$ + Vt2B

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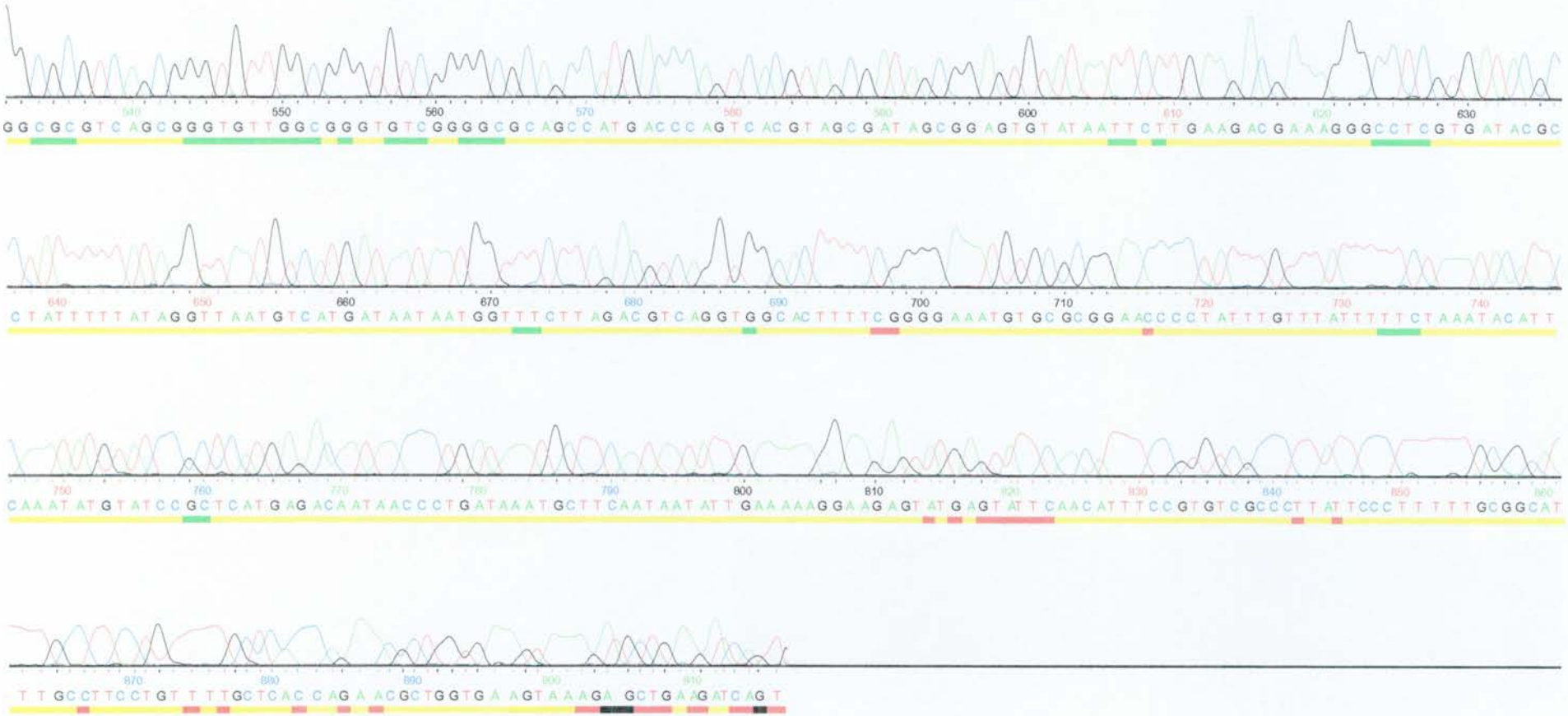

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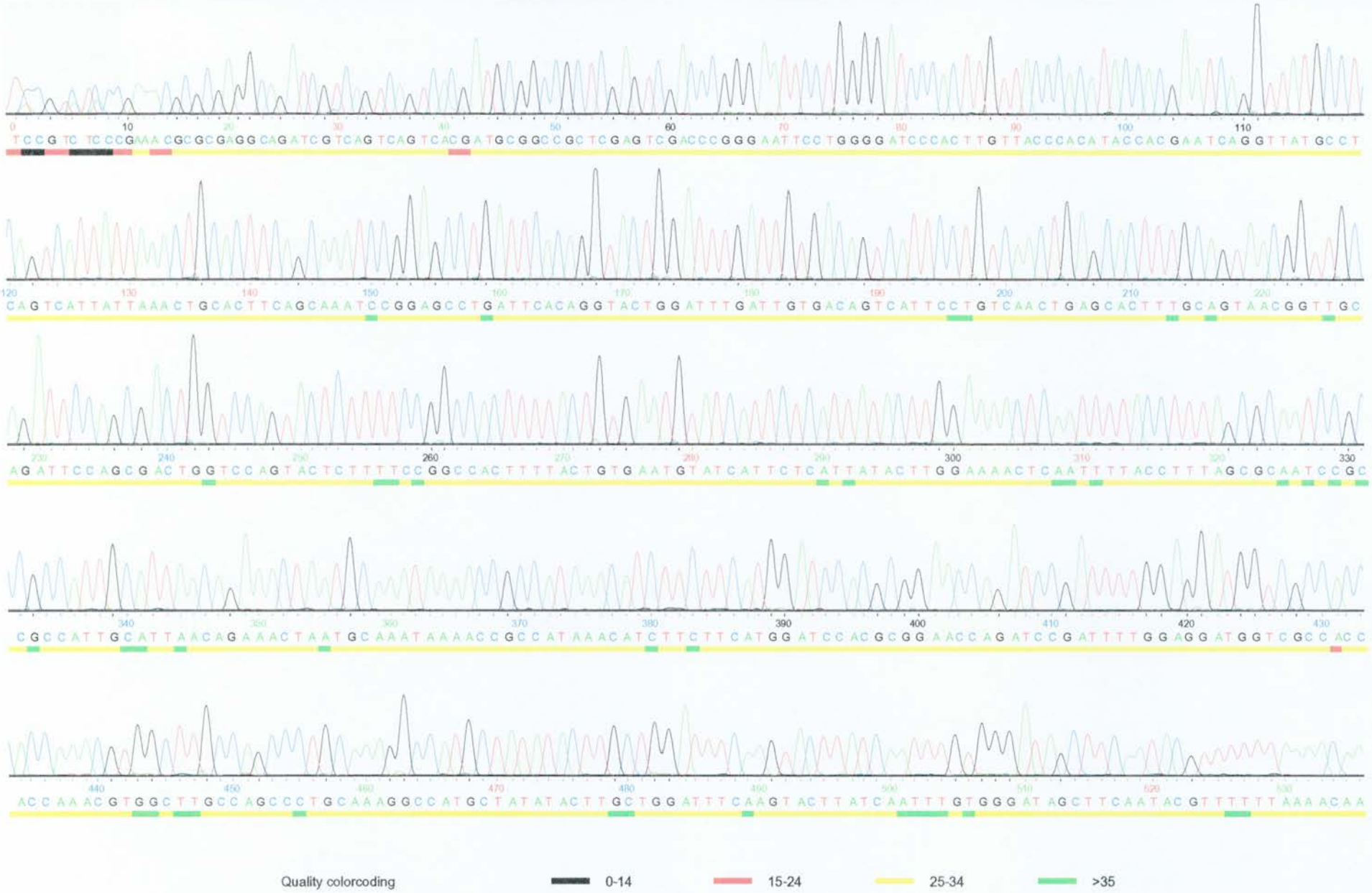
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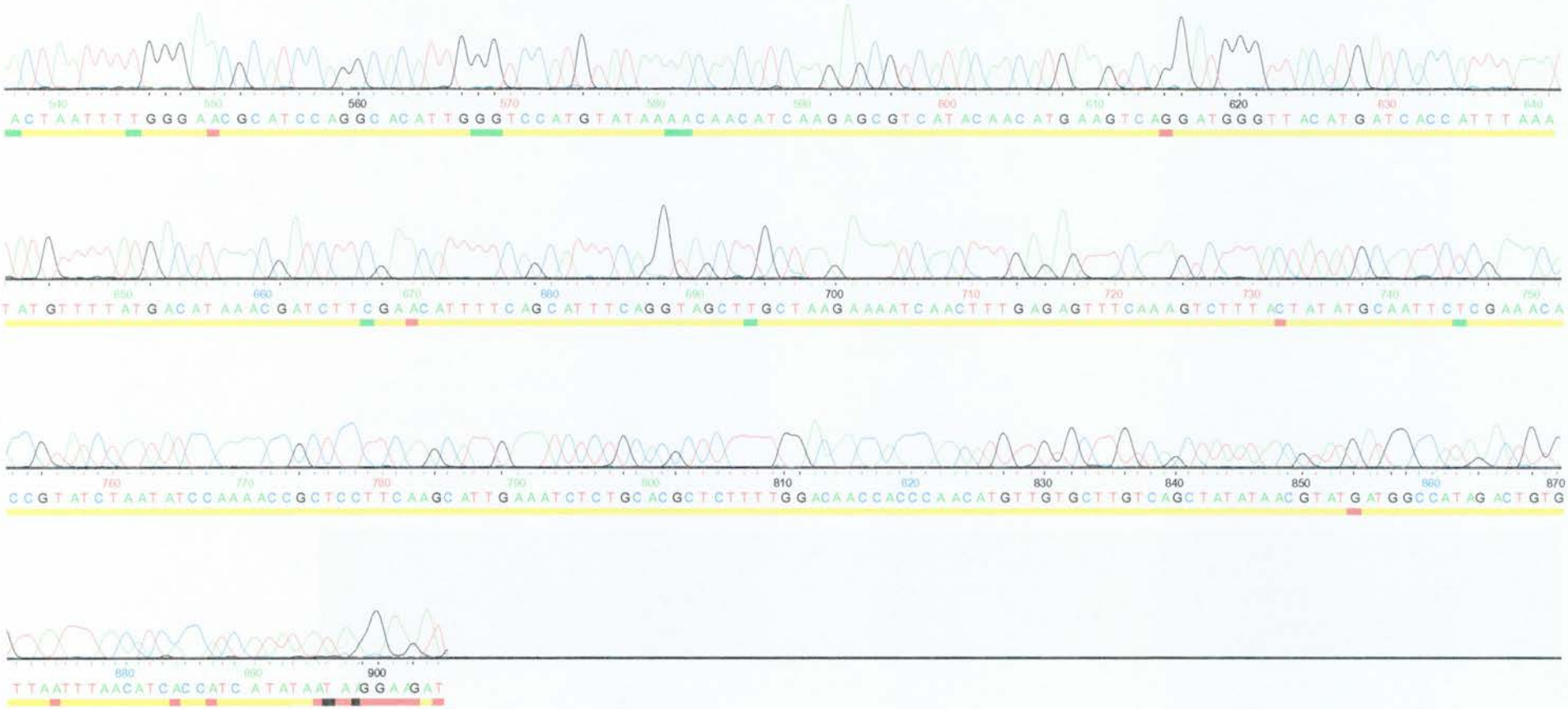
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>35

ClustalW Multiple Sequence Alignment Results : Verotoxin : pGEX for/rev sequences

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                    451         465 466         480 481         495 496         510 511         525 526         540
1 verotoxin         --------------- --------------- --------------- --------------- -------ATGAAGAA GATGTTTATGGCGGT     23
2 SampleVtBf-pGexfor -------CTGAGCCC GTTTGGTGGTGGCGA CCATCCTCCAAAATC GGATCTGGTTCCGCG TGGATCCATGAAGAA GATGTTTATGGCGGT     83
3 sampleVtBr-pGexrev GGGCTGGCAAGCCAC GTTTGGTGGTGGCGA CCATCCTCCAAAATC GGATCTGGTTCCGCG TGGATCCATGAAGAA GATGTTTATGGCGGT    540

                    541         555 556         570 571         585 586         600 601         615 616         630
1 verotoxin         TTTATTTGCATTAGC TTCTGTTAATGCAAT GGCGGCGGATTGTGC TAAAGGTAAAATTGA GTTTTCCAAGTATAA TGAGGATGACACATT    113
2 SampleVtBf-pGexfor TTTATTTGCATTAGT TTCTGTTAATGCAAT GGCGGCGGATTGCGC TAAAGGTAAAATTGA GTTTTCCAAGTATAA TGAGAATGATACATT    173
3 sampleVtBr-pGexrev TTTATTTGCATTAGT TTCTGTTAATGCAAT GGCGGCGGATTGCGC TAAAGGTAAAATTGA GTTTTCCAAGTATAA TGAGAATGATACATT    630

                    631         645 646         660 661         675 676         690 691         705 706         720
1 verotoxin         TACAGTGAAGGTTGA CGGGAAAGAATACTG GACCAGTCGCTGGAA TCTGCAACCGTTACT GCAAAGTGCTCAGTT GACAGGAATGACTGT    203
2 SampleVtBf-pGexfor CACAGTAAAAGTGGC CGGAAAAGAGTACTG GACCAGTCGCTGGAA TCTGCAACCGTTACT GCAAAGTGCTCAGTT GACAGGAATGACTGT    263
3 sampleVtBr-pGexrev CACAGTAAAAGTGGC CGGAAAAGAGTACTG GACCAGTCGCTGGAA TCTGCAACCGTTACT GCAAAGTGCTCAGTT GACAGGAATGACTGT    720

                    721         735 736         750 751         765 766         780 781         795 796         810
1 verotoxin         CACAATCAAATCCAG TACCTGTGAATCAGG CTCCGGATTTGCTGA AGTGCAGTTTAATAA TGACTGA-------- ---------------    270
2 SampleVtBf-pGexfor CACAATCAAATCCAG TACCTGTGAATCAGG CTCCGGATTTGCTGA AGTGCAGTTTAATAA TGACTGAGGCATAAC CTGATTCGTGGTATG    353
3 sampleVtBr-pGexrev CACAATCAAATCCAG TACCTGTGAATCAGG CTCCGGATTTGCTGA AGTGCAGTTTAATAA TGACTGAGGCATAAC CTGATTCGTGGTATG    810

                    811         825 826         840 841         855 856         870 871         885 886         900
1 verotoxin         --------------- --------------- --------------- --------------- --------------- ---------------    270
2 SampleVtBf-pGexfor TGGGTAACAAGTGGG ATCCCCAGGAATTCC CGGGTCGACTCGAGC GGCCGCATCGTGACT GACTGACGATCTGCC TCGCGCGTTTCGGTG    443
3 sampleVtBr-pGexrev TGGGTAACAAGTGGG ATCCCCAGGAATTCC CGGGTCGACTCGAGC GGCCGCATCGTGACT GACTGACGATCTGCC TCGCGCGTTTCGGG-    899

                    901         915 916         930 931         945 946         960 961         975 976         990
1 verotoxin         --------------- --------------- --------------- --------------- --------------- ---------------    270
2 SampleVtBf-pGexfor ATGACGGTGAAAACC TCTGACACATGCAGC TCCCGGAGACGGTCA CAGCTTGTCTGTAAG CGGATGCCGGGAGCA GACAAGCCCGTCAGG    533
3 sampleVtBr-pGexrev -AGACGGA------- --------------- --------------- --------------- --------------- ---------------    906
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Appendix 3: Immunoblots

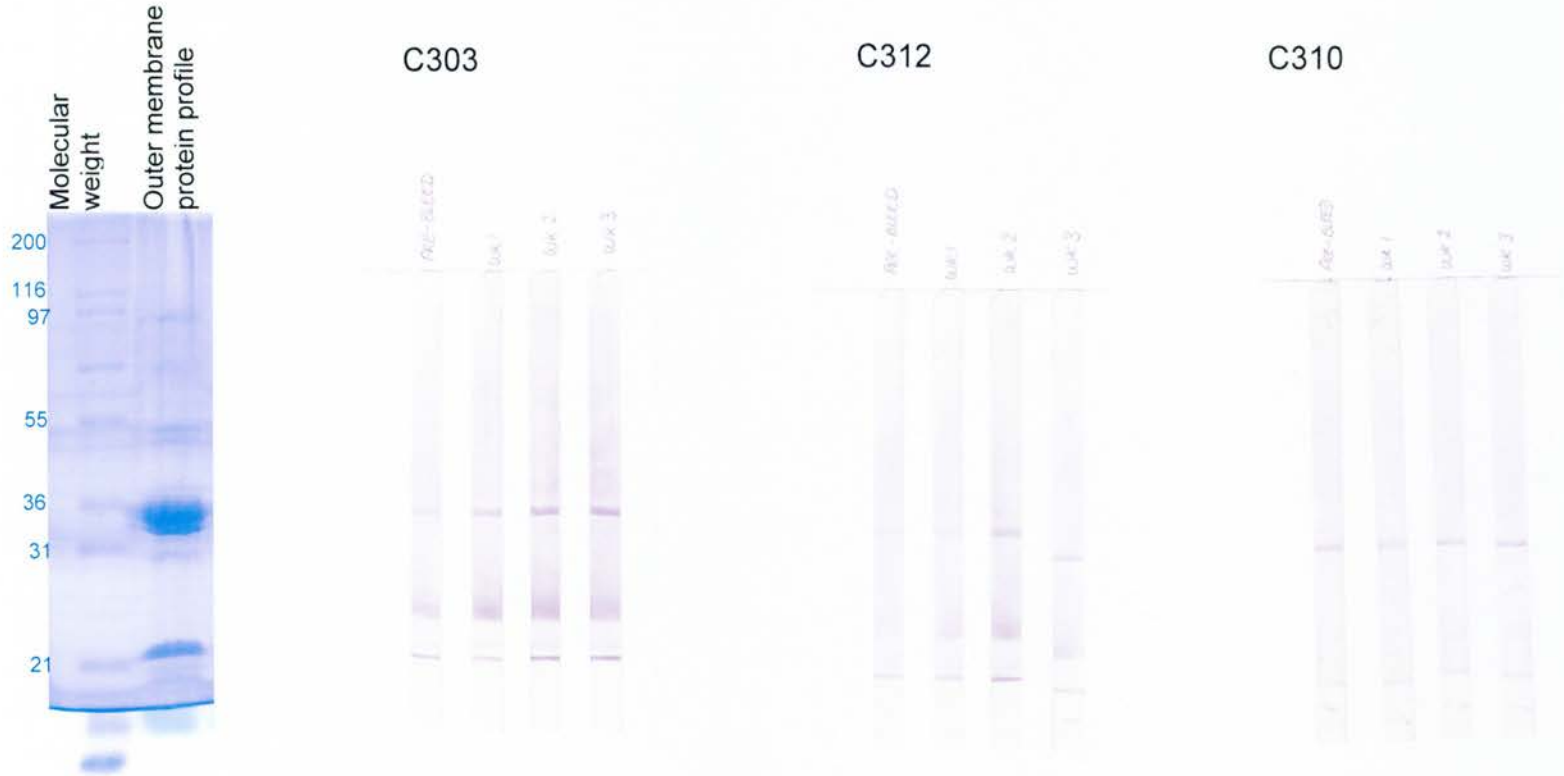


Figure A3.1. Antibody responses to the outer membrane proteins of *E. coli* O157**.** Outer membrane preparations from a toxin negative *E.coli* O157 resolved by 10% SDS-PAGE and transferred to nitrocellulose for immunoblot. The first blot in each series of four shows the pre-bleed IgG status of each of the four calves (c303, c312 and c315). Each blot thereafter (wk1, wk2 and wk3) represents the immune status at weekly intervals after the initial challenge. Note sera from the fourth week of the experiment were not available. A 10% SDS-PAGE outer membrane protein profile (left hand side) along with the molecular weight of the proteins is shown for reference.

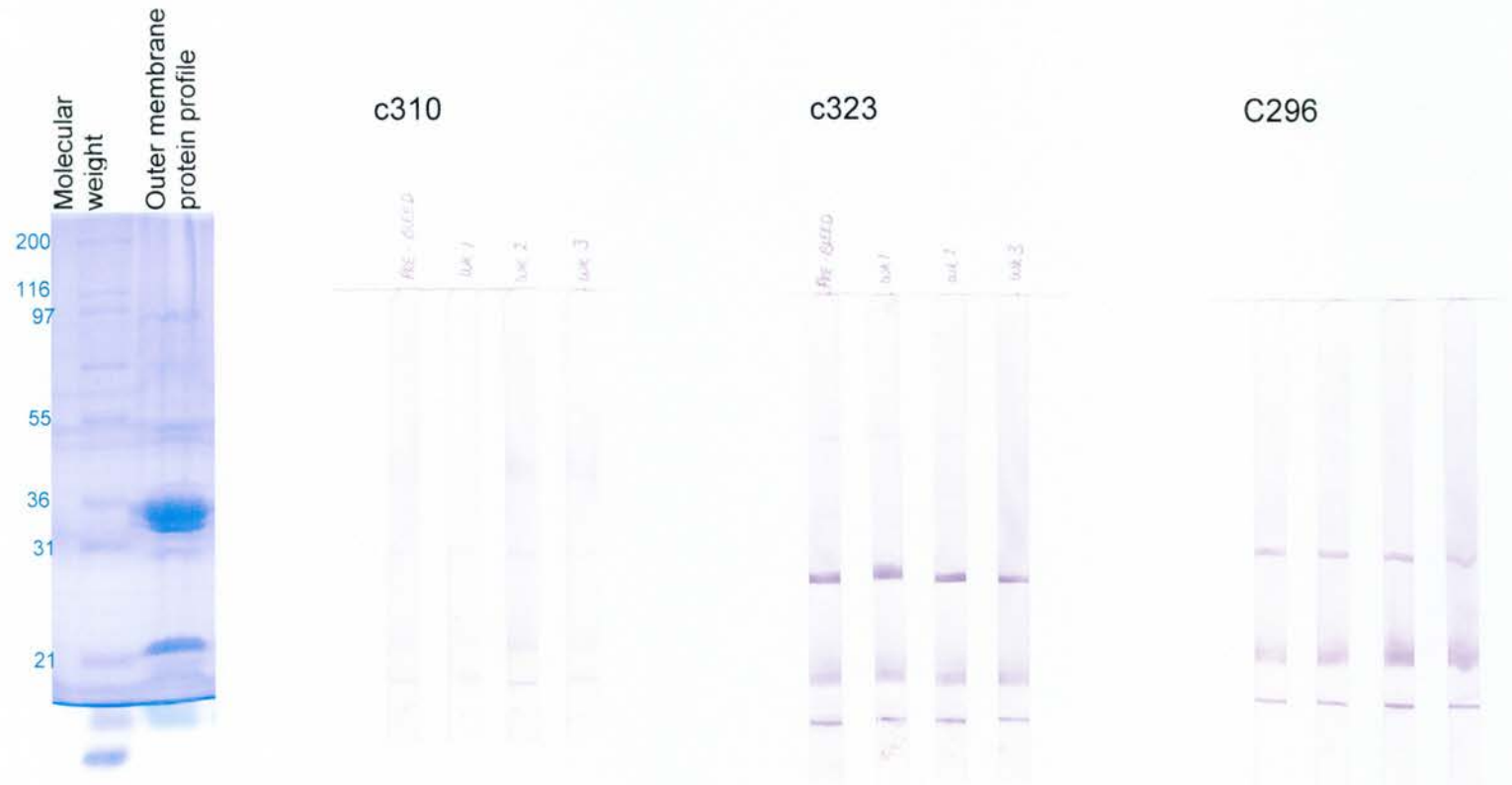


Figure A3.2. Antibody responses to the outer membrane proteins of *E. coli* O157**.** Outer membrane preparations from a toxin negative *E.coli* O157 resolved by 10% SDS-PAGE and transferred to nitrocellulose for immunoblot. The first blot in each series of four shows the pre-bleed IgG status of each of the four calves (c310, c323, and c296). Each blot thereafter (wk1, wk2 and wk3) represents the immune status at weekly intervals after the initial challenge. Note sera from the fourth week of the experiment were not available. A 10% SDS-PAGE outer membrane protein profile (left hand side) along with the molecular weight of the proteins is shown for reference.

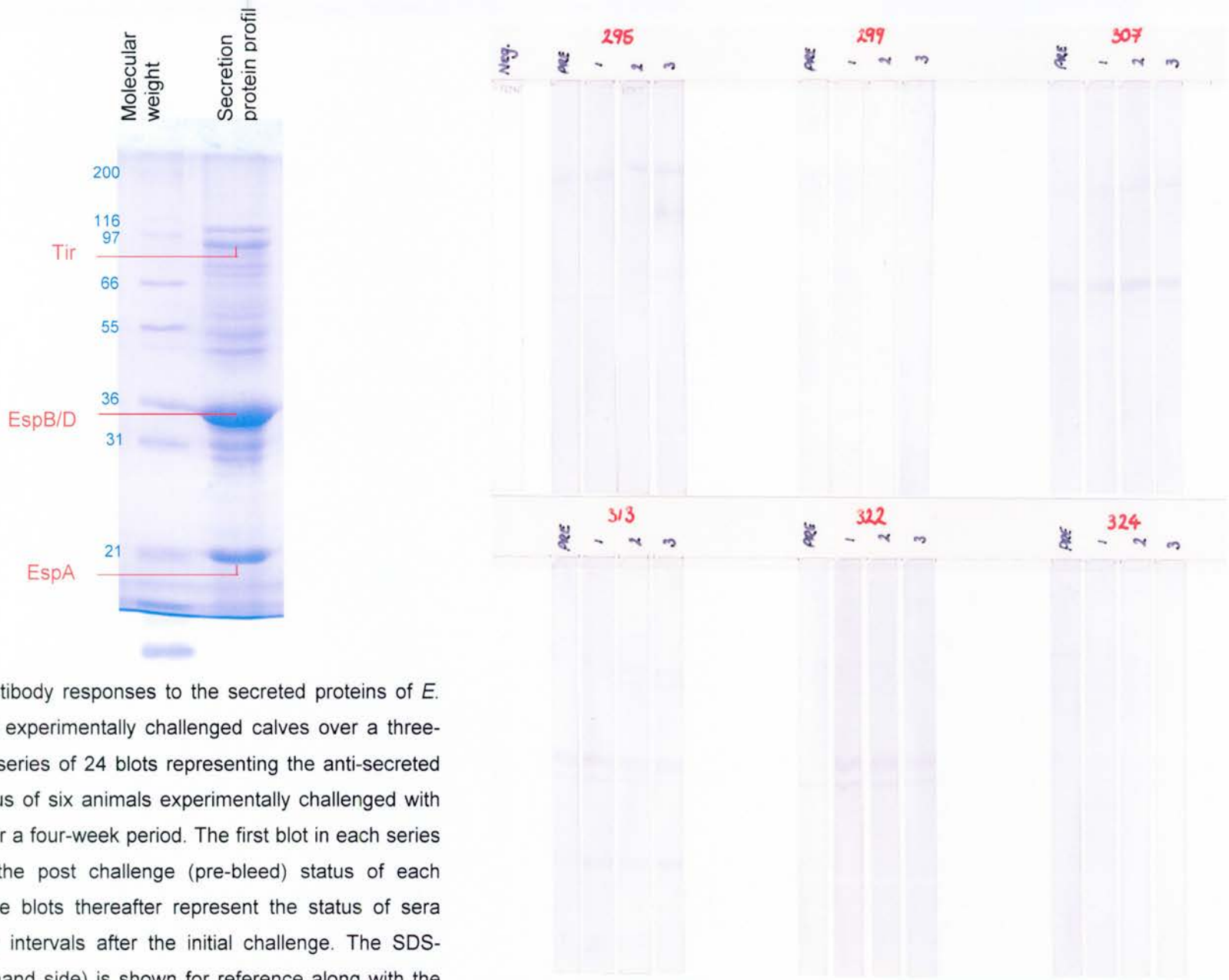


Figure A3.3. Antibody responses to the secreted proteins of *E. coli* O157 in six experimentally challenged calves over a three-week period. A series of 24 blots representing the anti-secreted protein IgG status of six animals experimentally challenged with *E. coli* O157 over a four-week period. The first blot in each series of four shows the post challenge (pre-bleed) status of each animal, the three blots thereafter represent the status of sera taken at weekly intervals after the initial challenge. The SDS-PAGE gel (left hand side) is shown for reference along with the molecular weight markers. Proteins of interest are noted at the side. Sera from week four were not available.

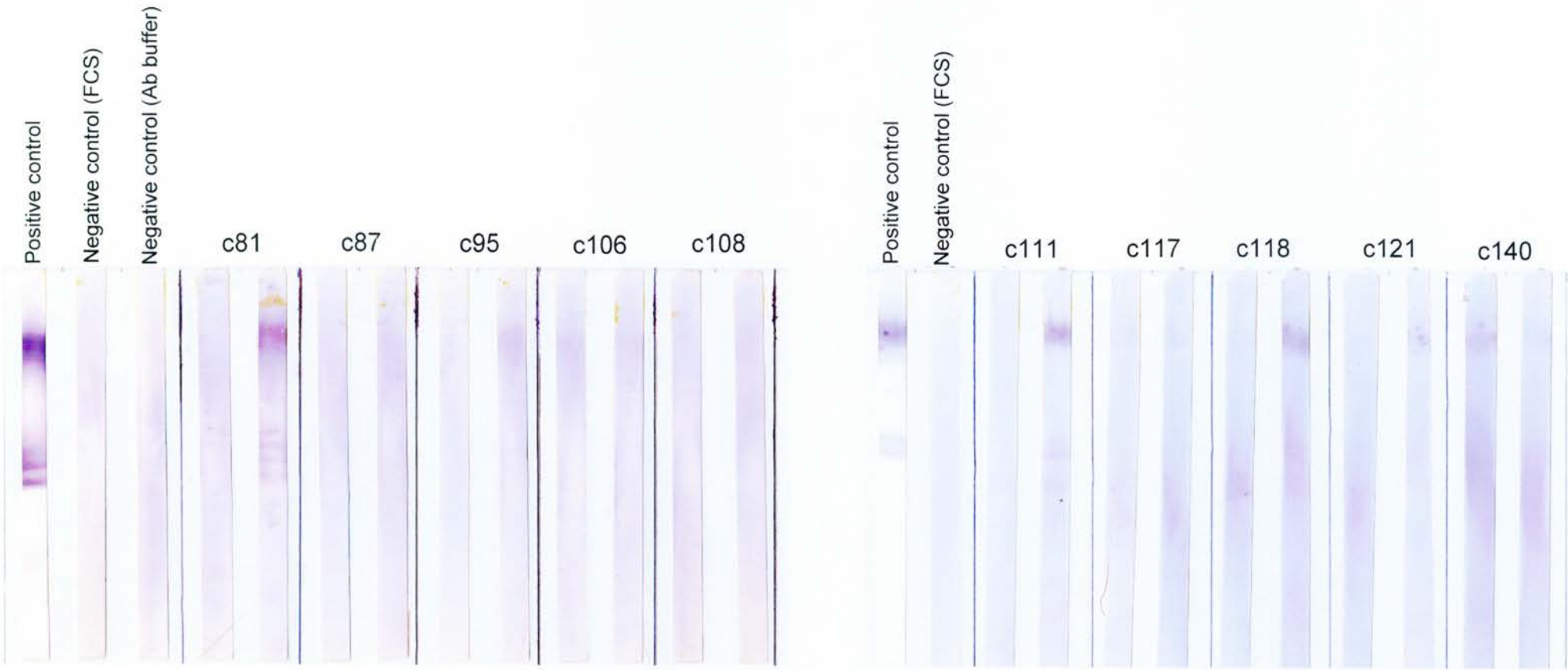


Figure A3.4. The response to *E. coli* O157 LPS in 10 experimentally infected calves over a four-week period. Each pair of blots shows the post challenge and week four post challenge status of each animal. The positive control (generated with sera from a rabbit known to have anti-*E. coli* O157 LPS antibodies) and the negative controls (generated using foetal calf serum and antibody buffer) are shown.

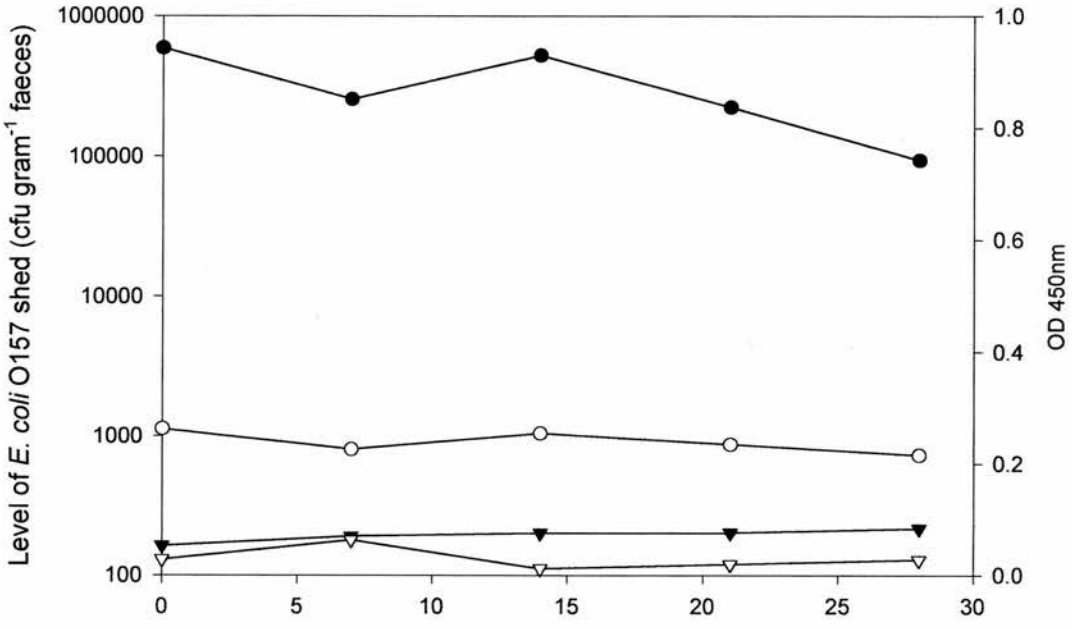
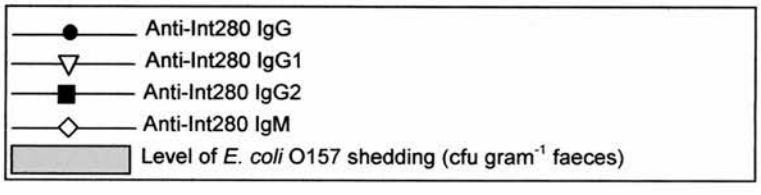


Figure A4.1: Calf c81. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-Int280 immunoglobulin over the same period (right axis).



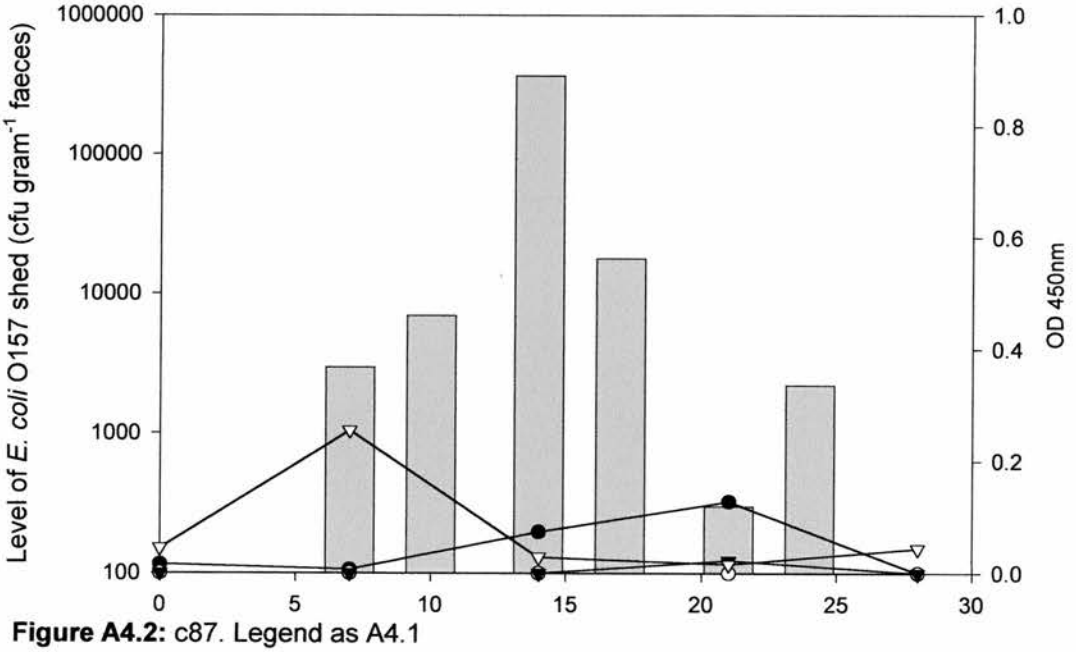


Figure A4.2: c87. Legend as A4.1

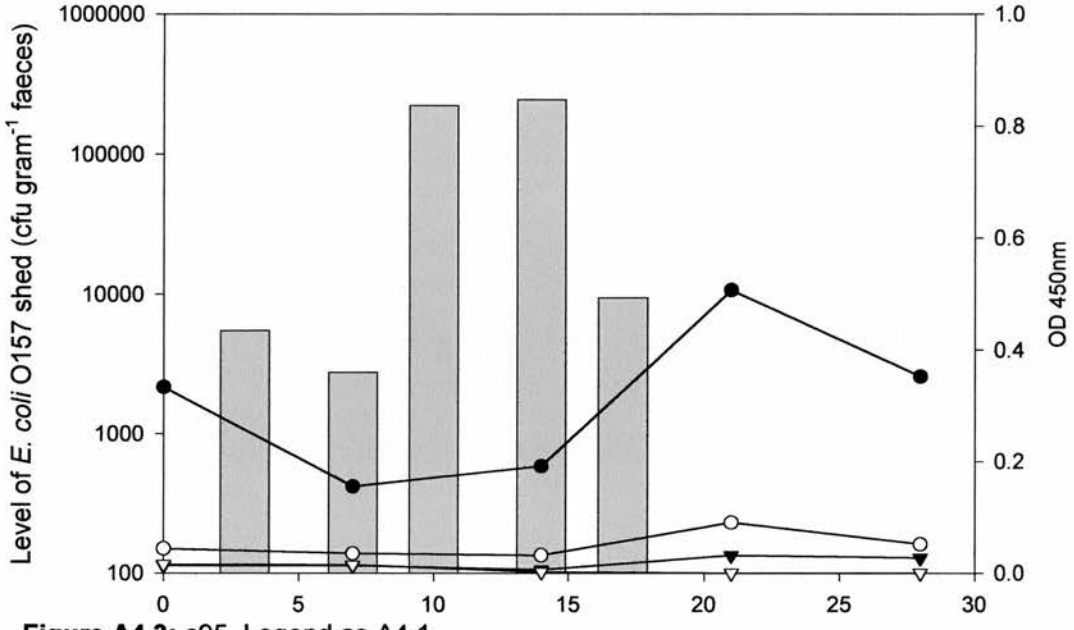


Figure A4.3: c95. Legend as A4.1

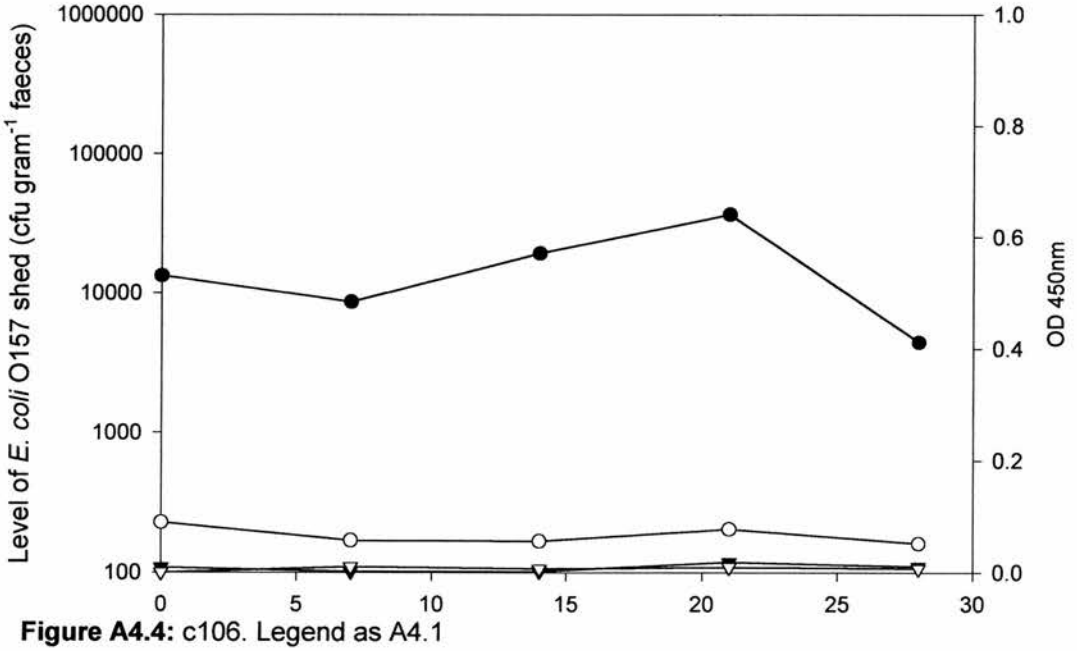


Figure A4.4: c106. Legend as A4.1

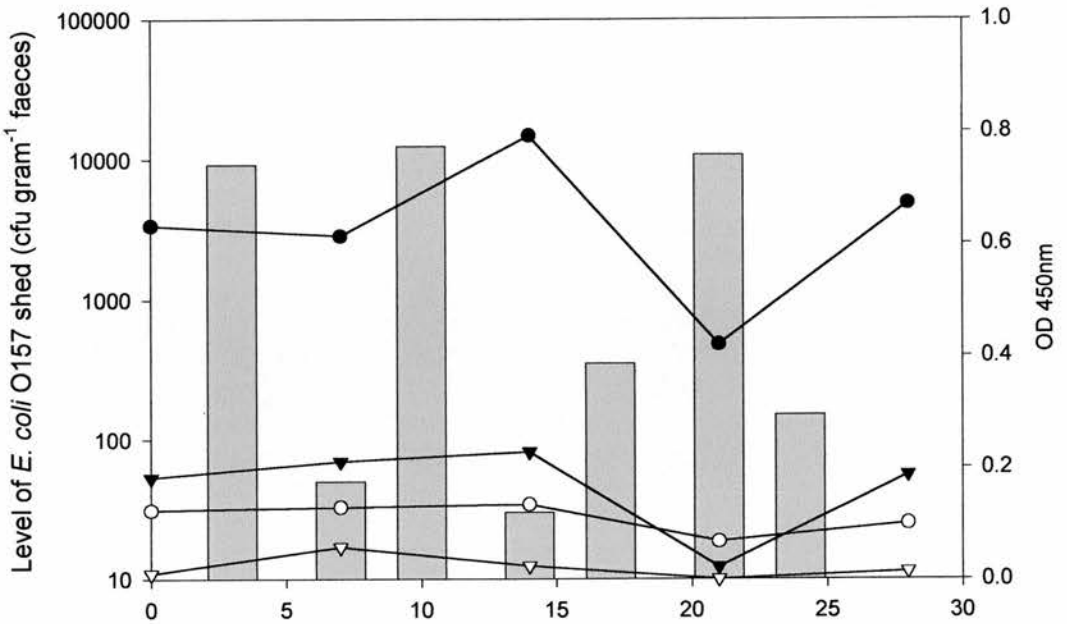


Figure A4.5: c108. Legend as A4.1

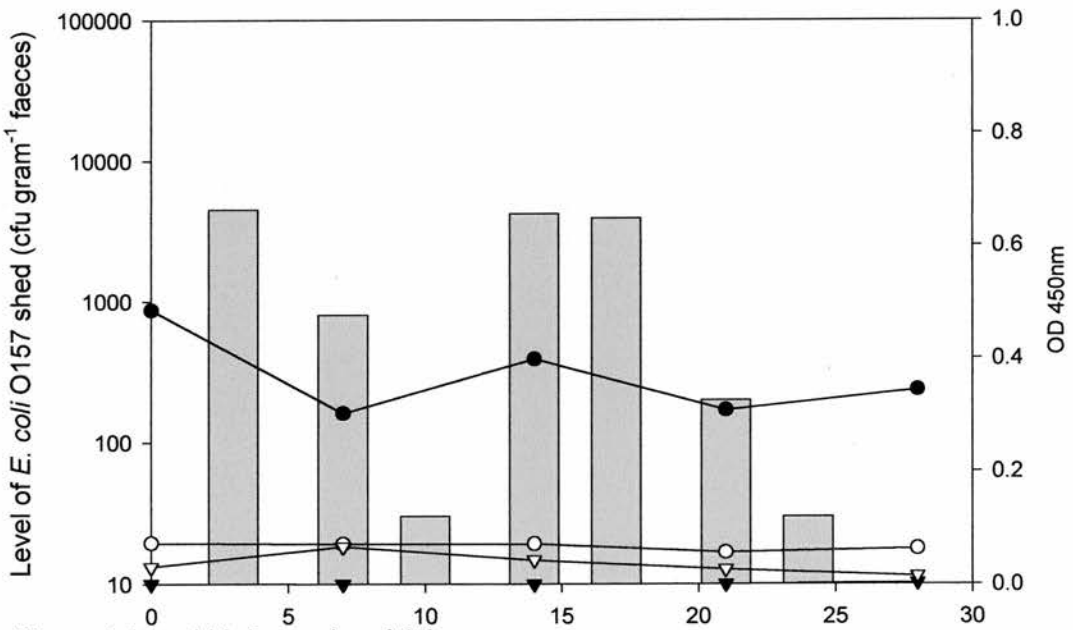


Figure A4.6: c111. Legend as A4.1

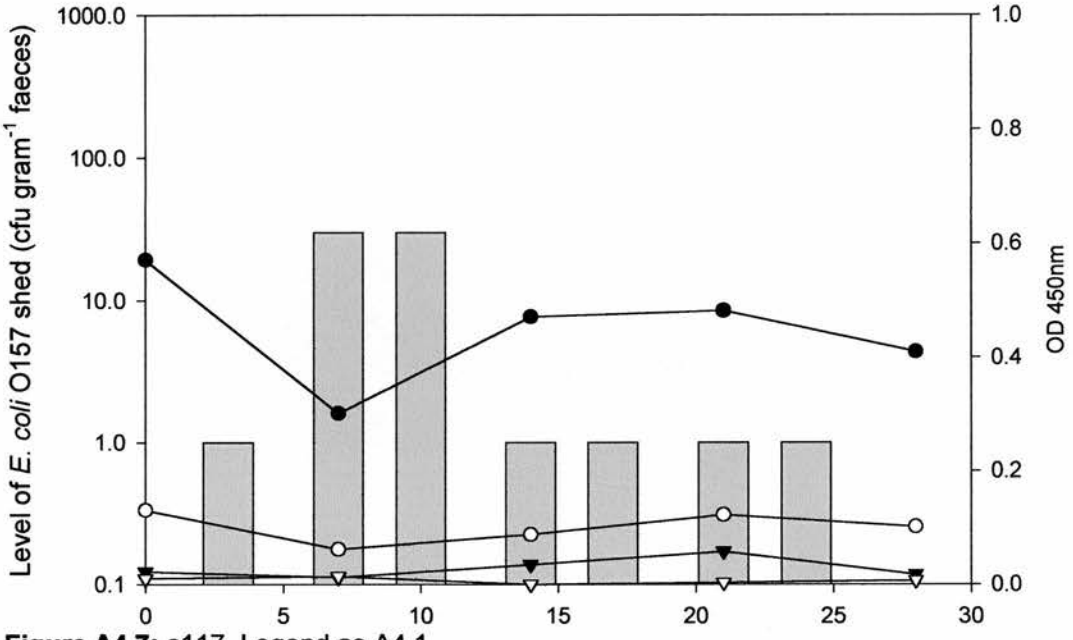


Figure A4.7: c117. Legend as A4.1

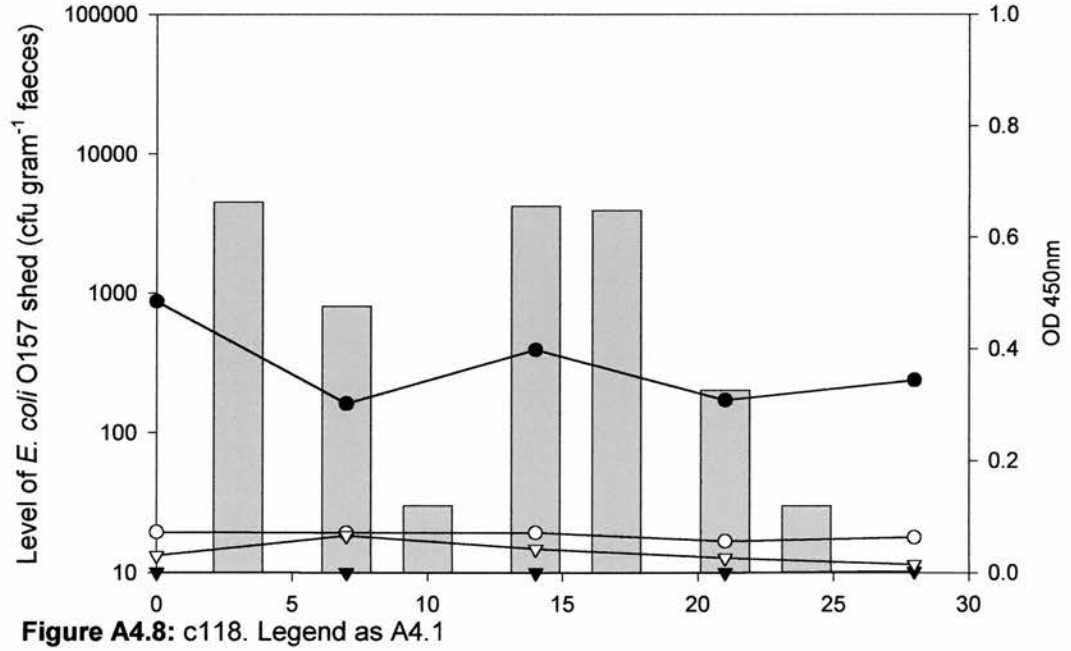


Figure A4.8: c118. Legend as A4.1

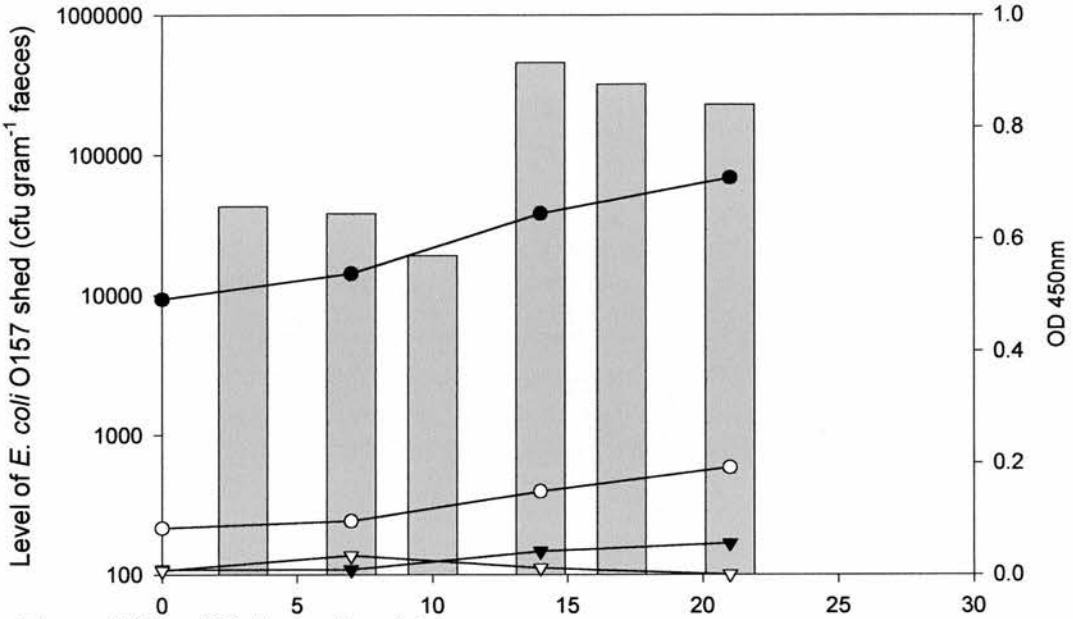


Figure A4.9: c121. Legend as A4.1

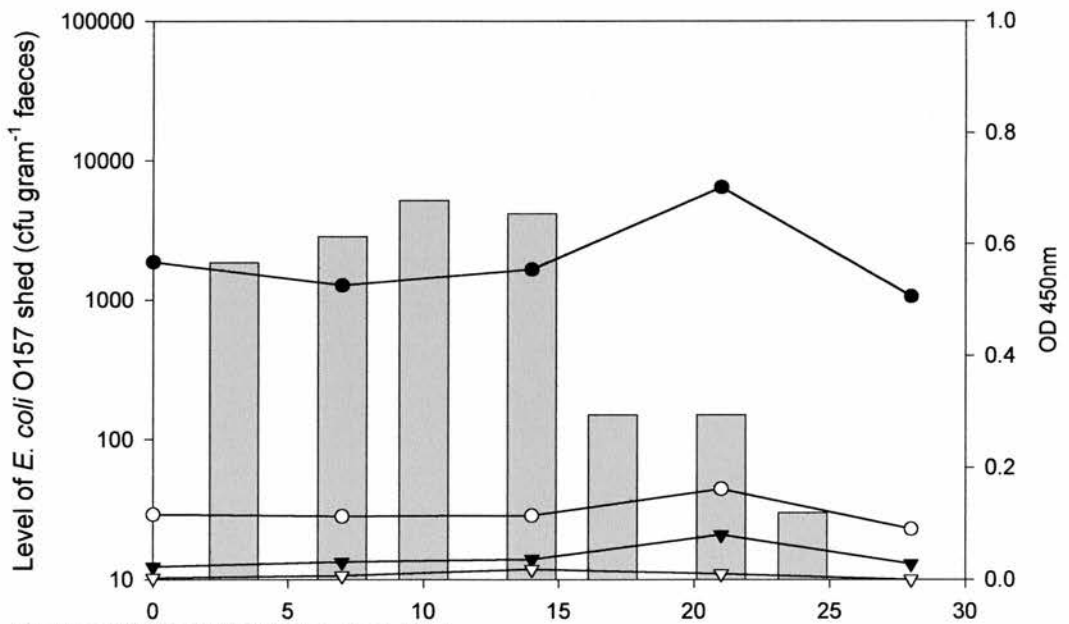


Figure A4.10: c140. Legend as A4.1

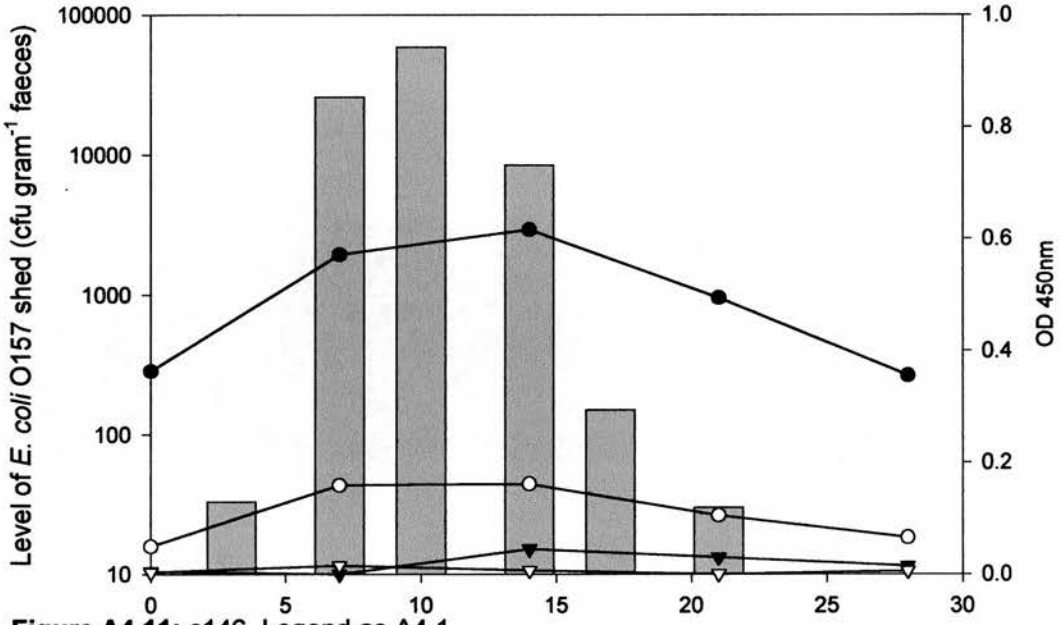


Figure A4.11: c146. Legend as A4.1

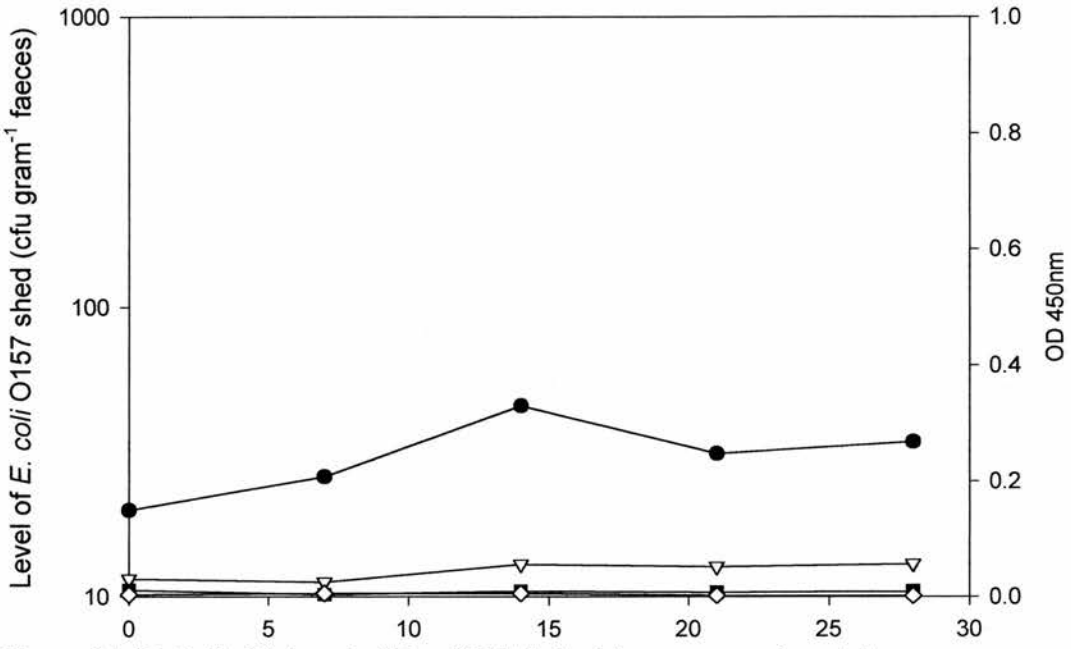


Figure A4.12: Calf c81. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-EspB immunoglobulin over the same period (right axis).

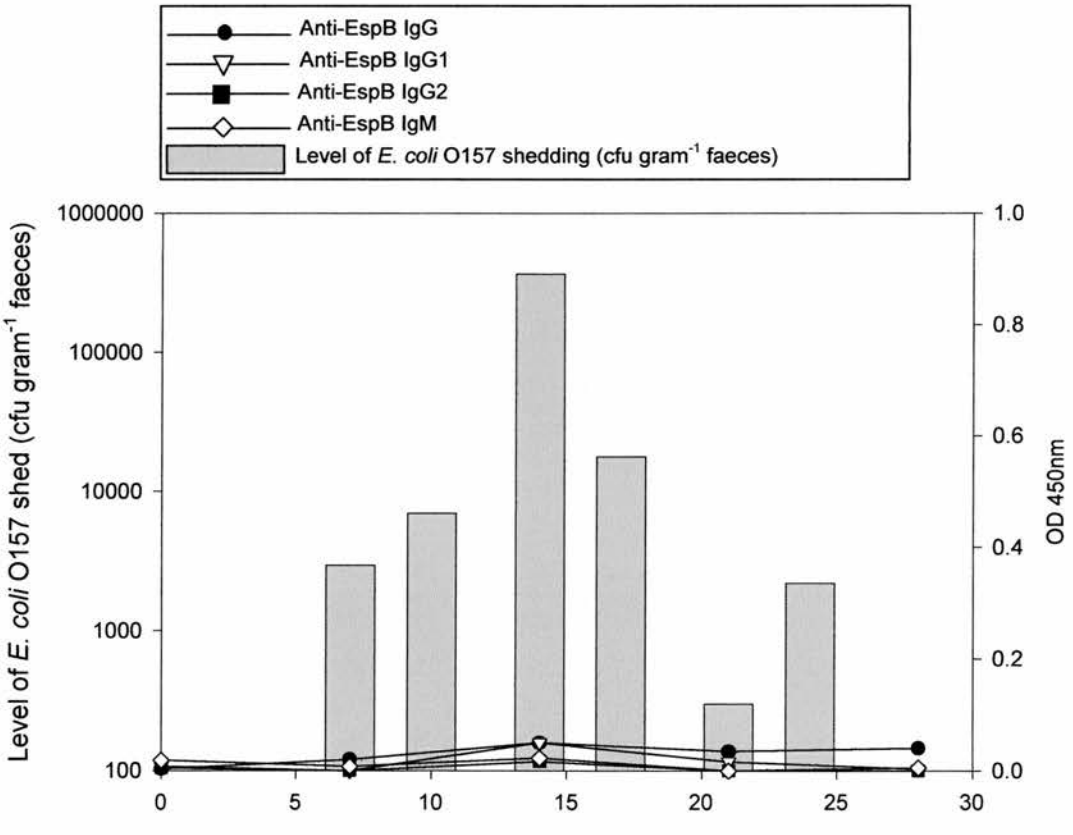


Figure A4.13: c87. Legend as A4.12

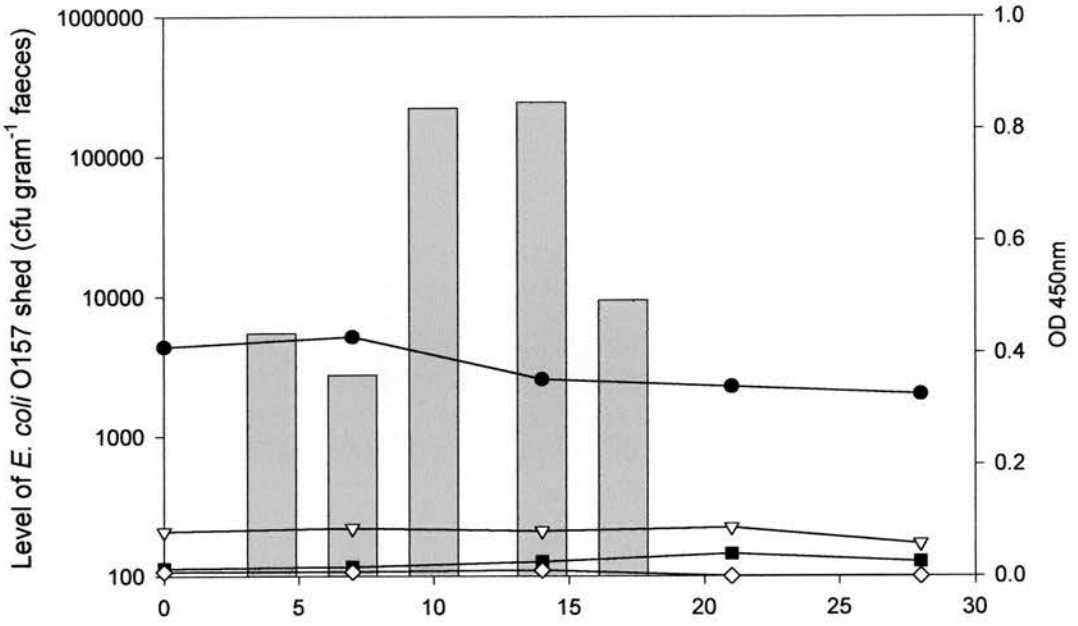


Figure A4.14: c95. Legend as A4.12

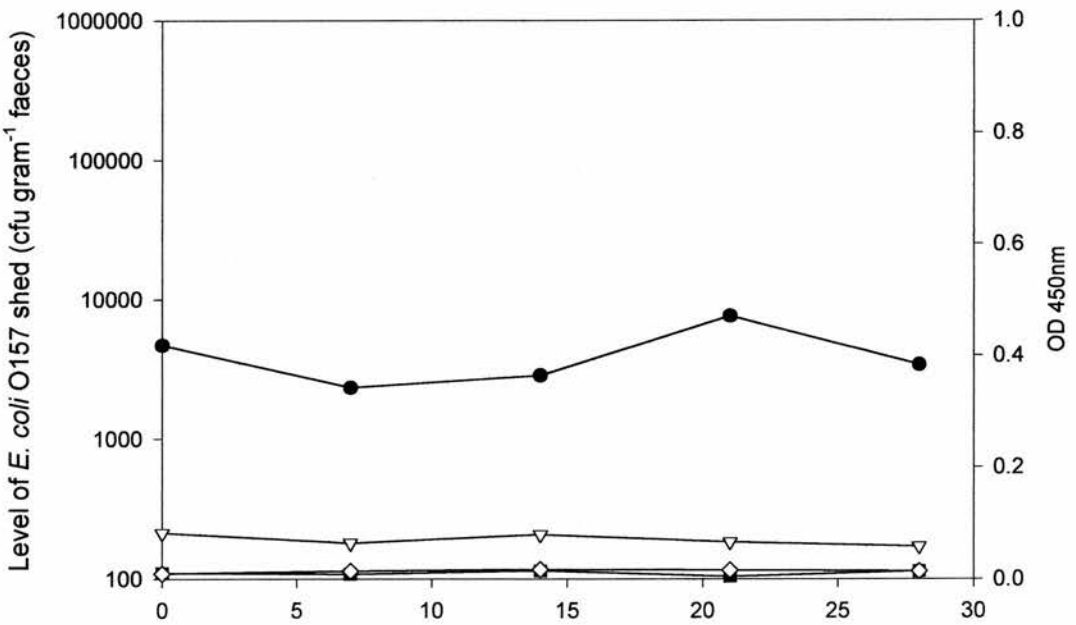


Figure A4.15: c106. Legend as A4.12

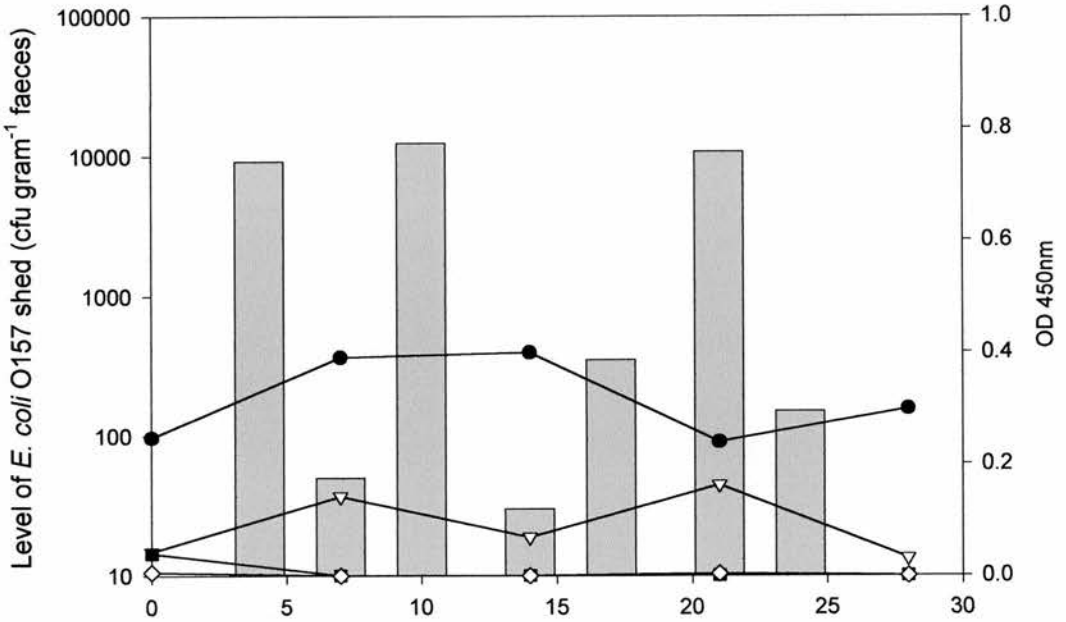


Figure A4.16: c108. Legend as A4.12

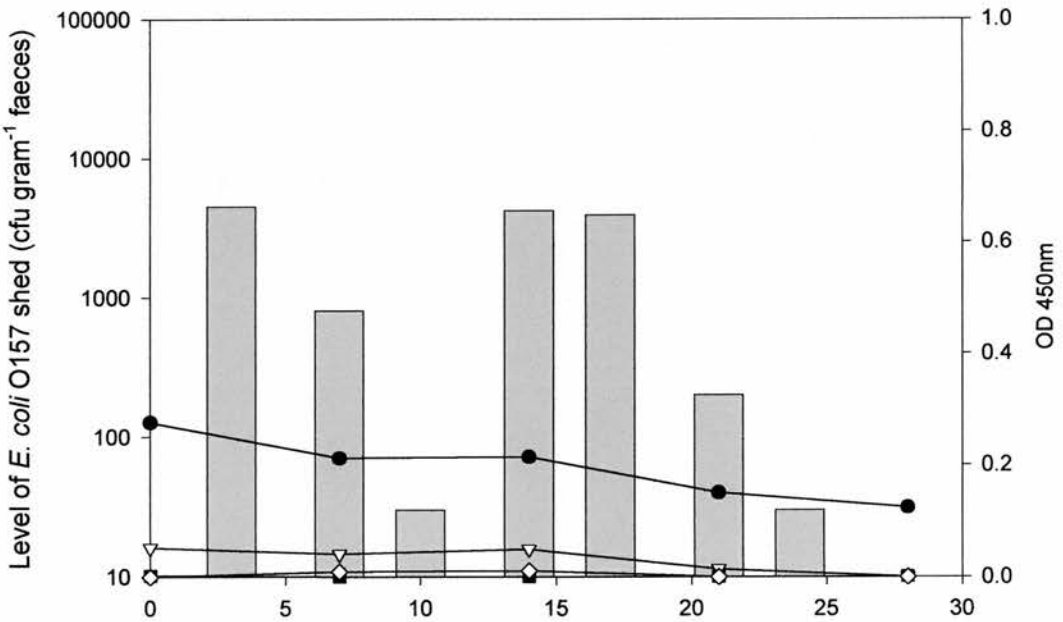


Figure A4.17: c111. Legend as A4.12

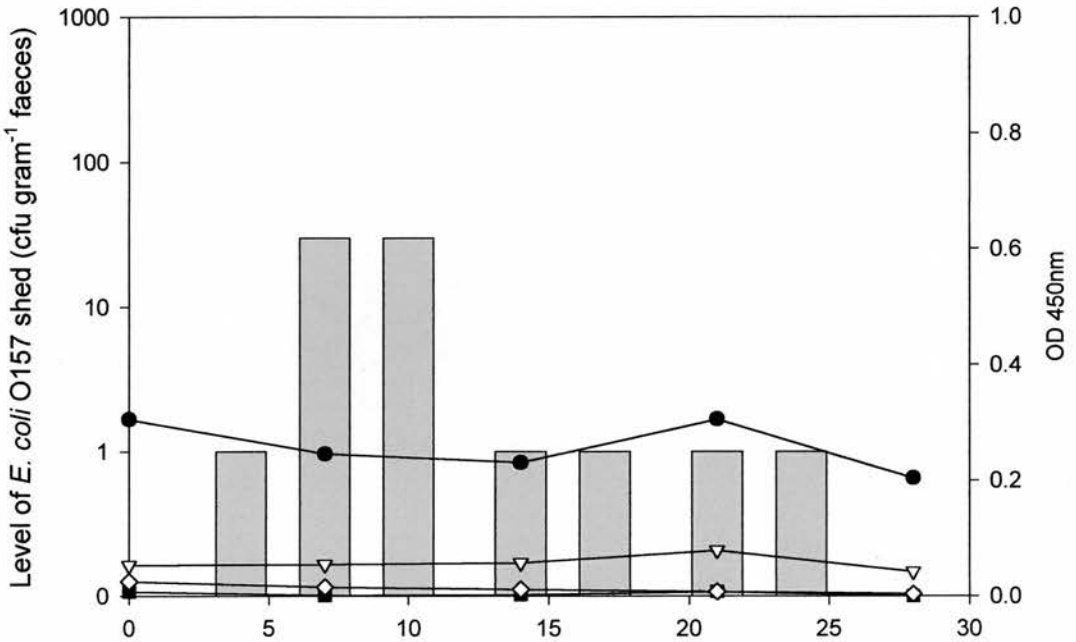


Figure A4.18: c117. Legend as A4.12

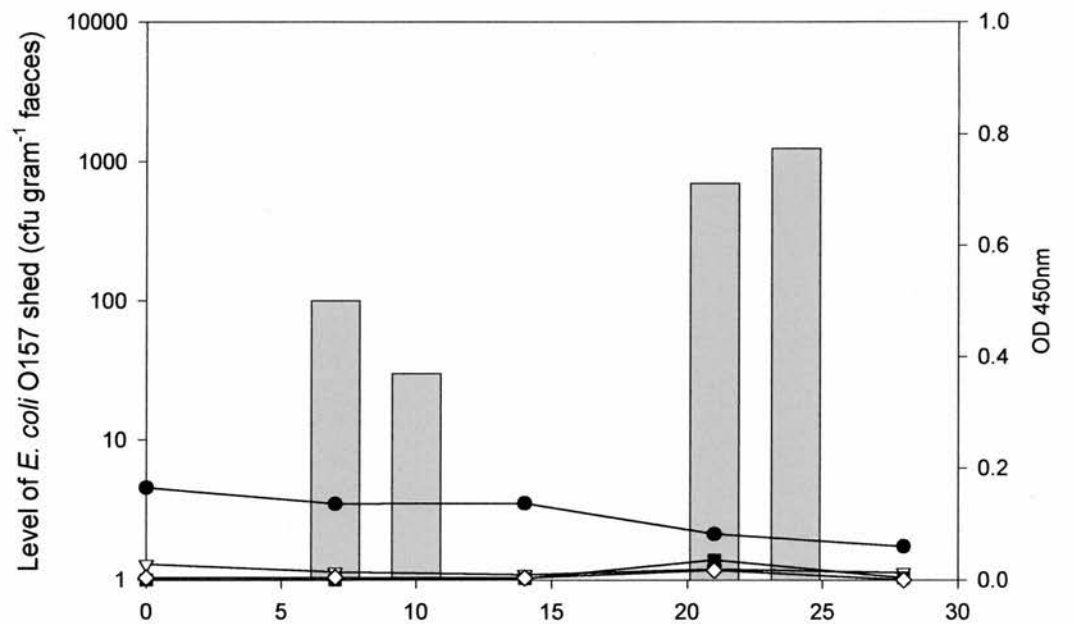


Figure 4.19: c118. Legend as A4.12

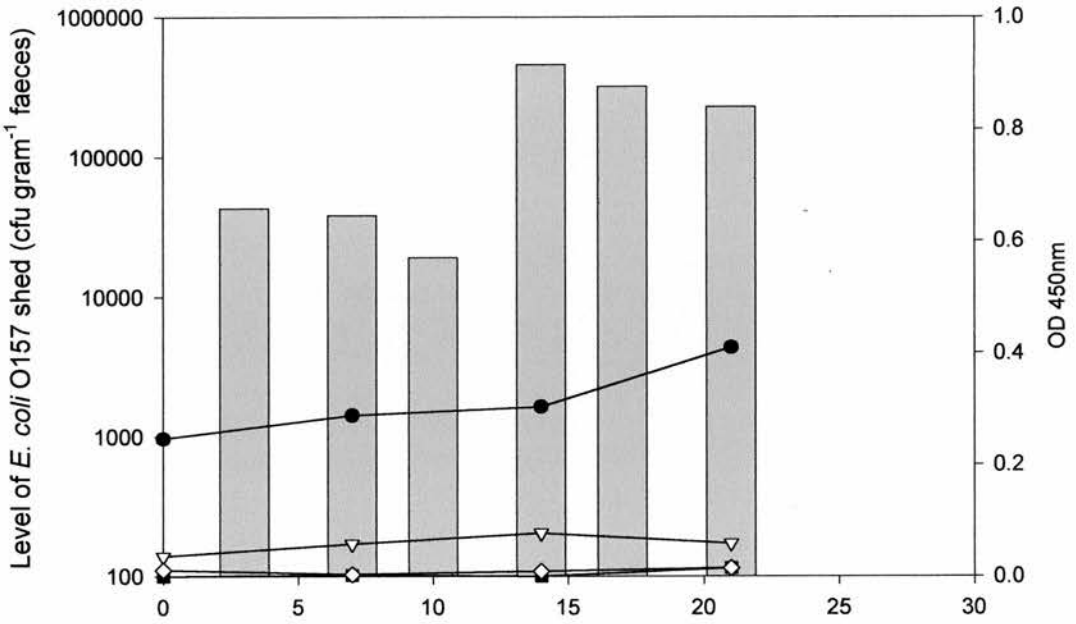


Figure A4.20: c121. Legend as A4.12

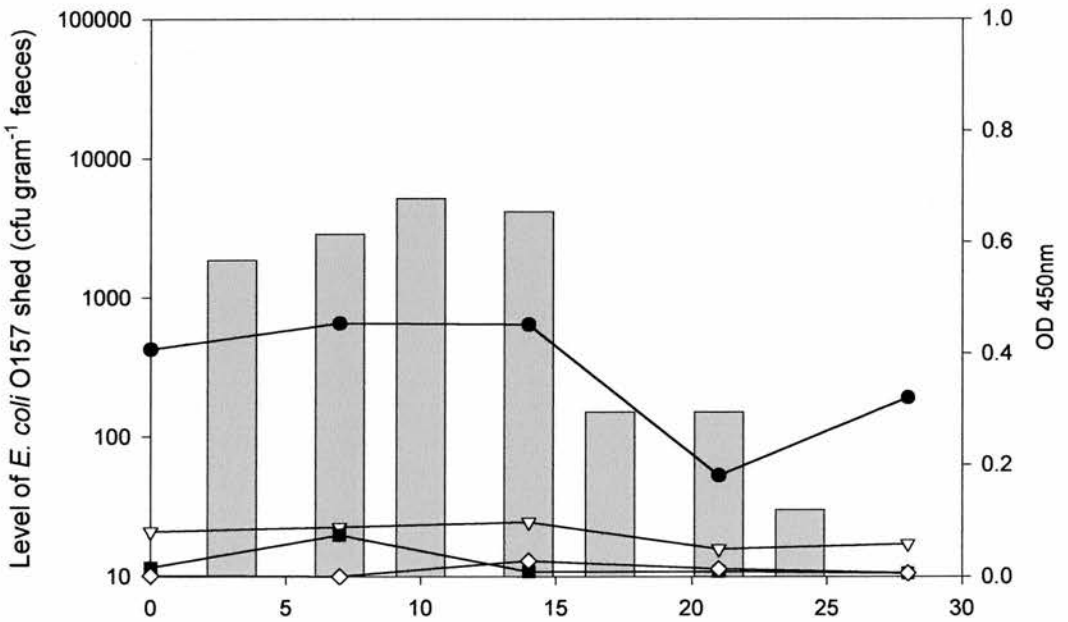


Figure A4.21: c140. Legend as A4.12

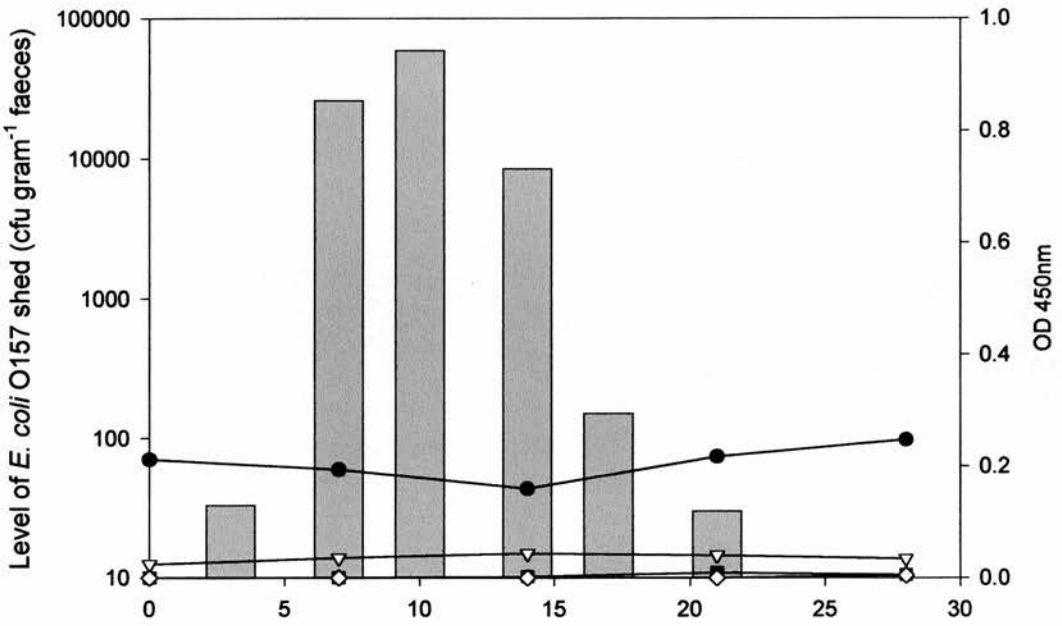


Figure A4.22: c146. Legend as A4.12

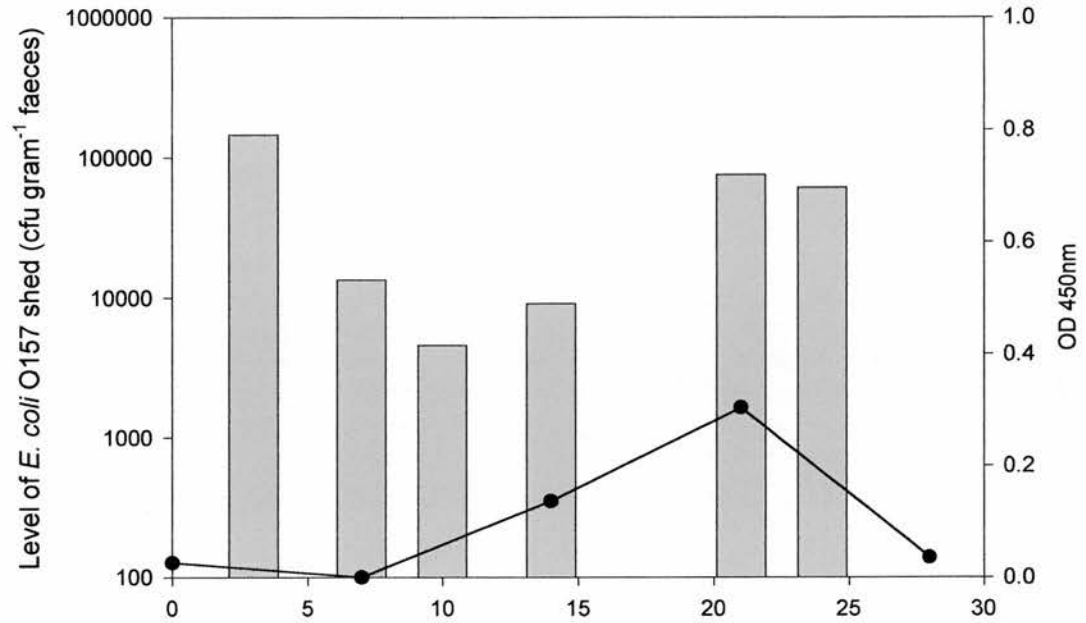
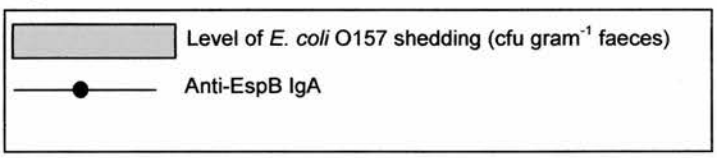


Figure A4.23: Calf c295. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-EspB IgA over the same period (right axis).



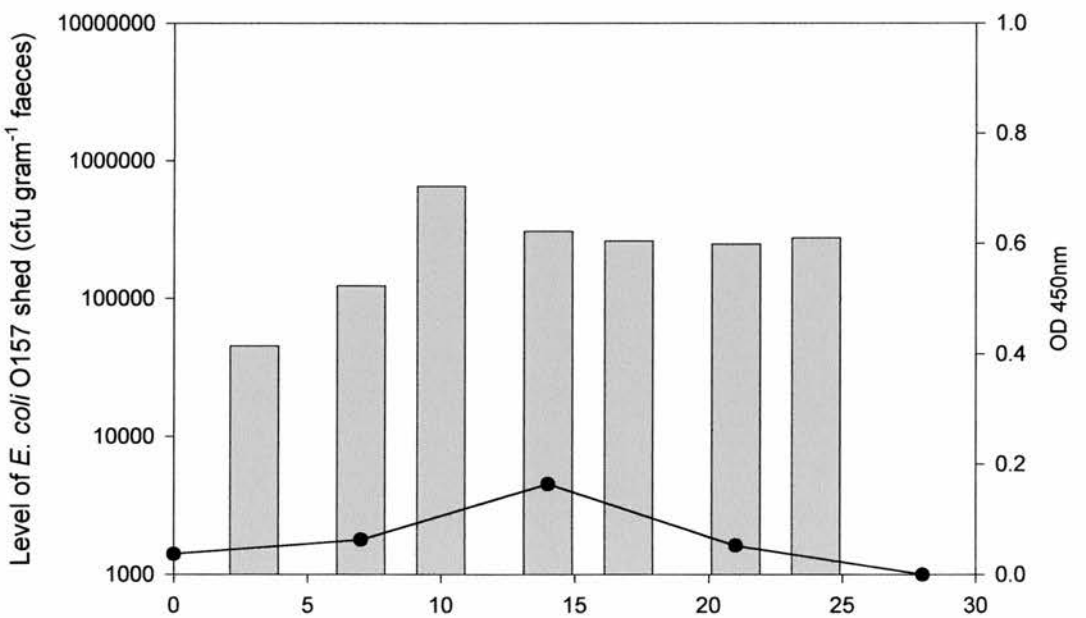


Figure A4.24: Calf c299. Legend as A4.23

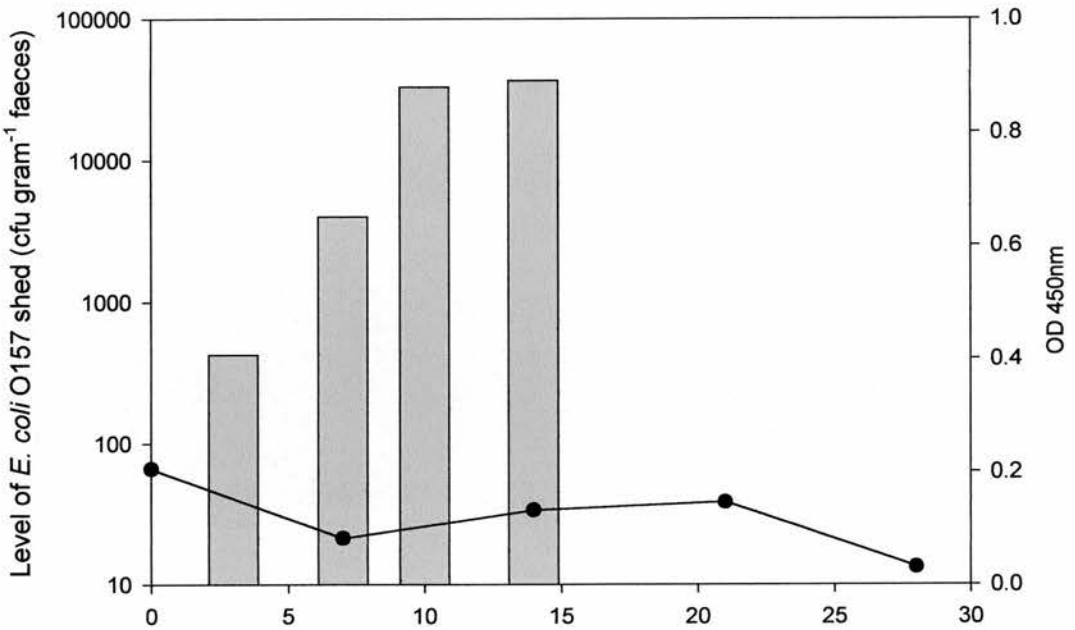


Figure A4.25: Calf c307. Legend as A4.23

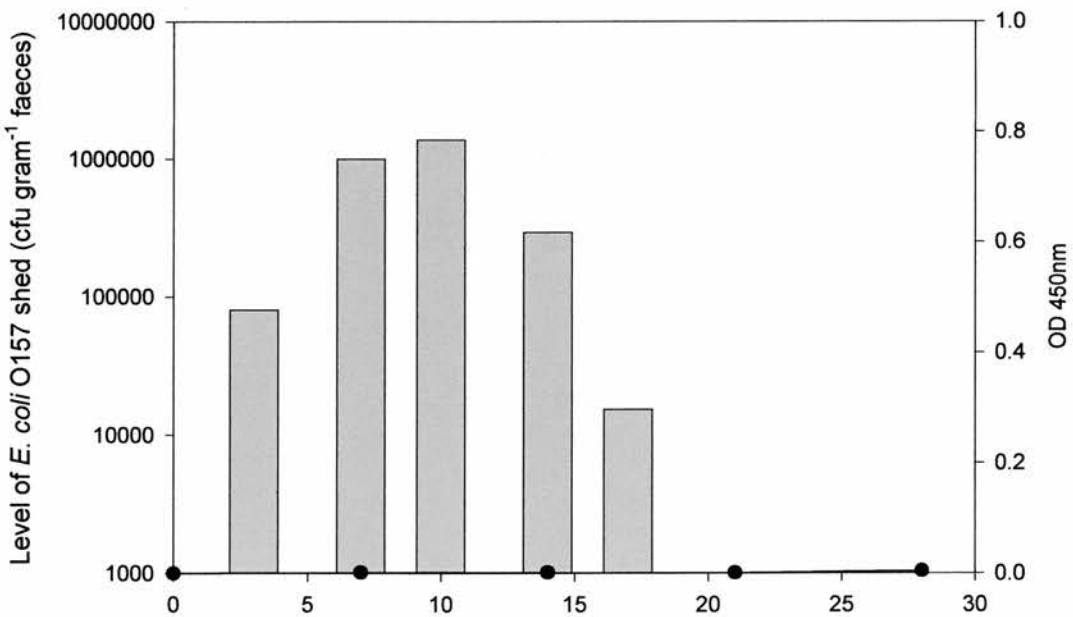


Figure A4.26: Calf c313. Legend as A4.23

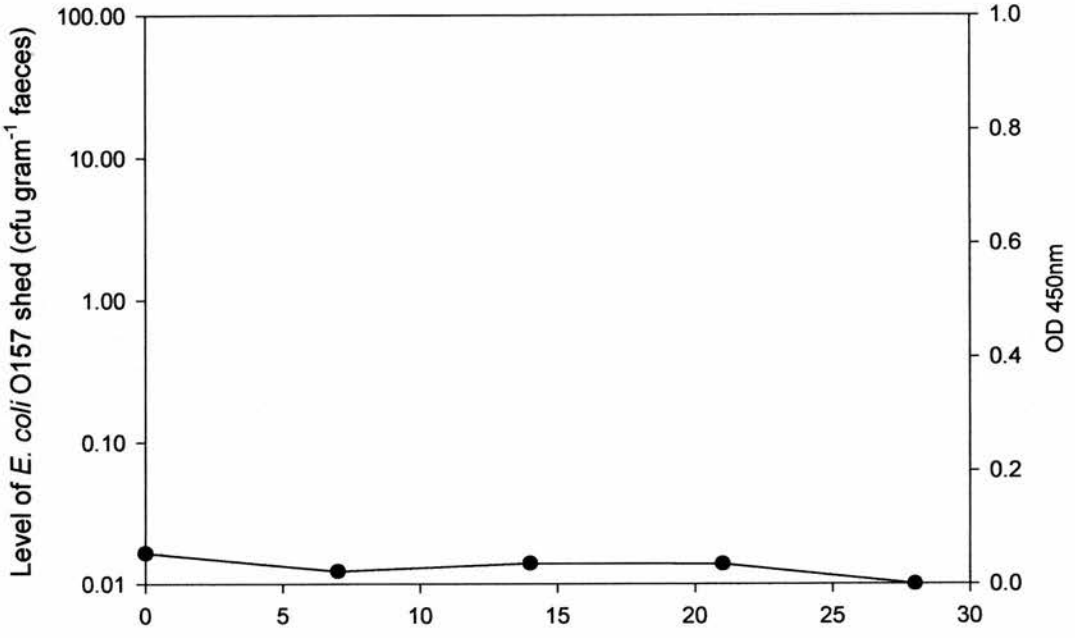


Figure A4.27: Calf c322. Legend as A4.23

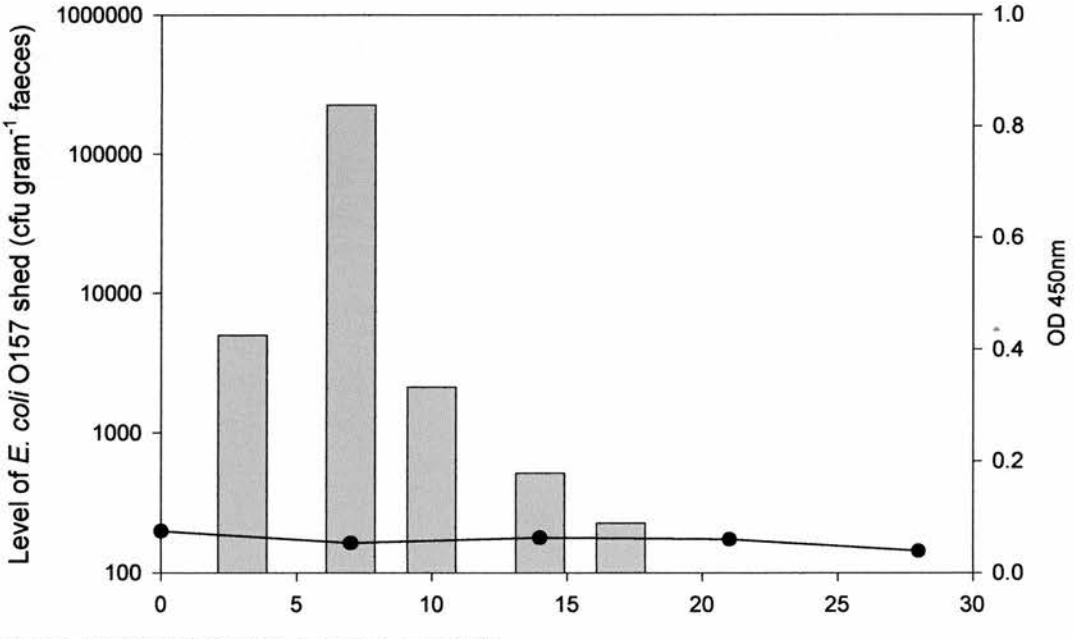


Figure A4.28: Calf c324. Legend as A4.23

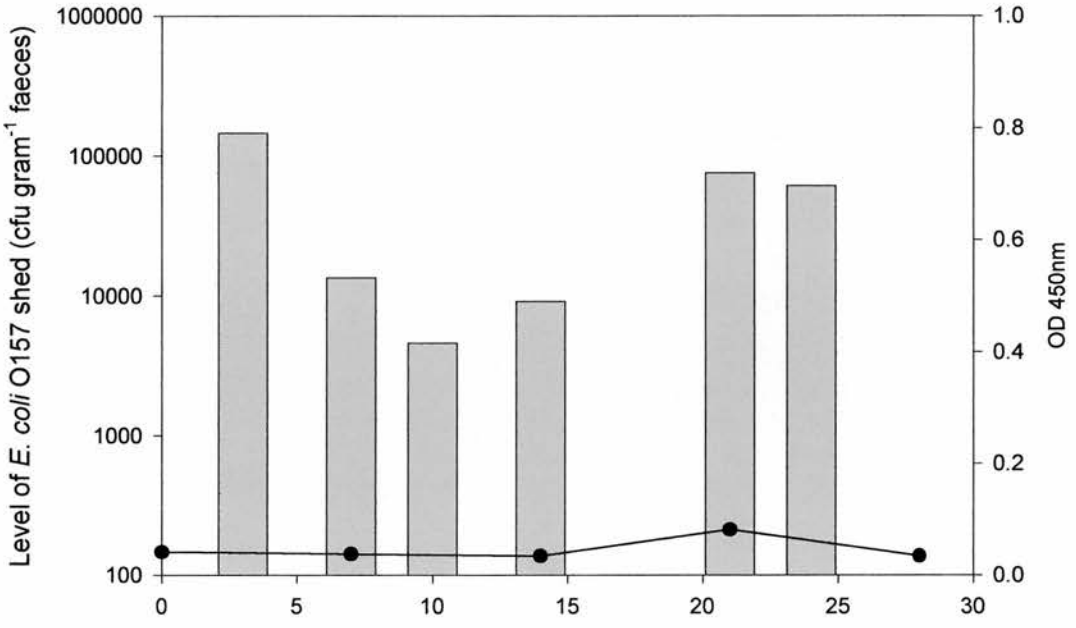
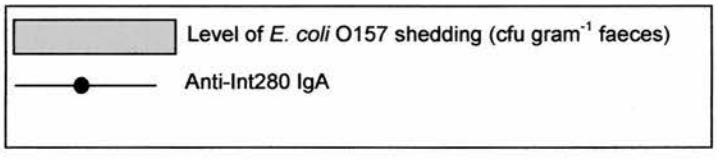


Figure A4.29: Calf c295. Level of *E. coli* O157 shed from an experimentally infected calf over a four week period (left axis), and the level of anti-INT280 IgA over the same period (right axis).



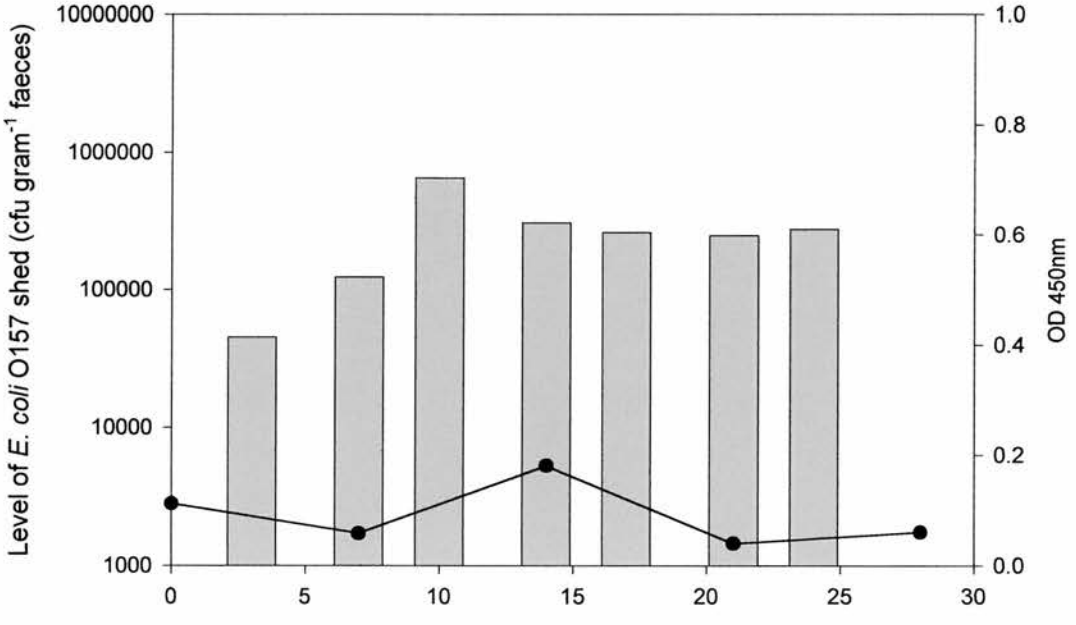


Figure A4.30: Calf c299. Legend as A4.29

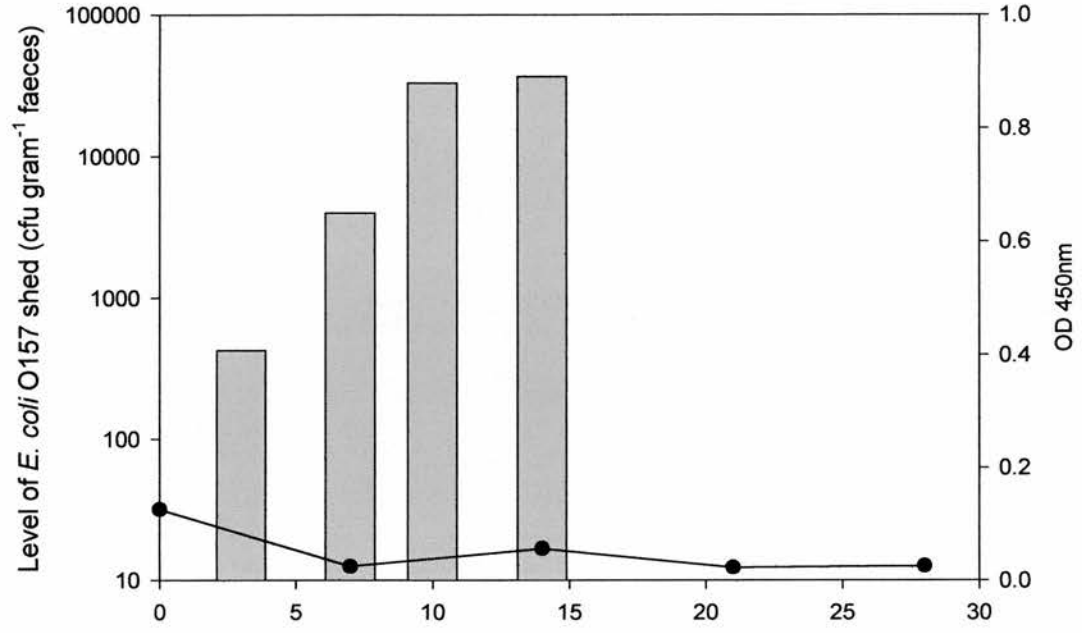


Figure A4.31: Calf c307. Legend as A4.29

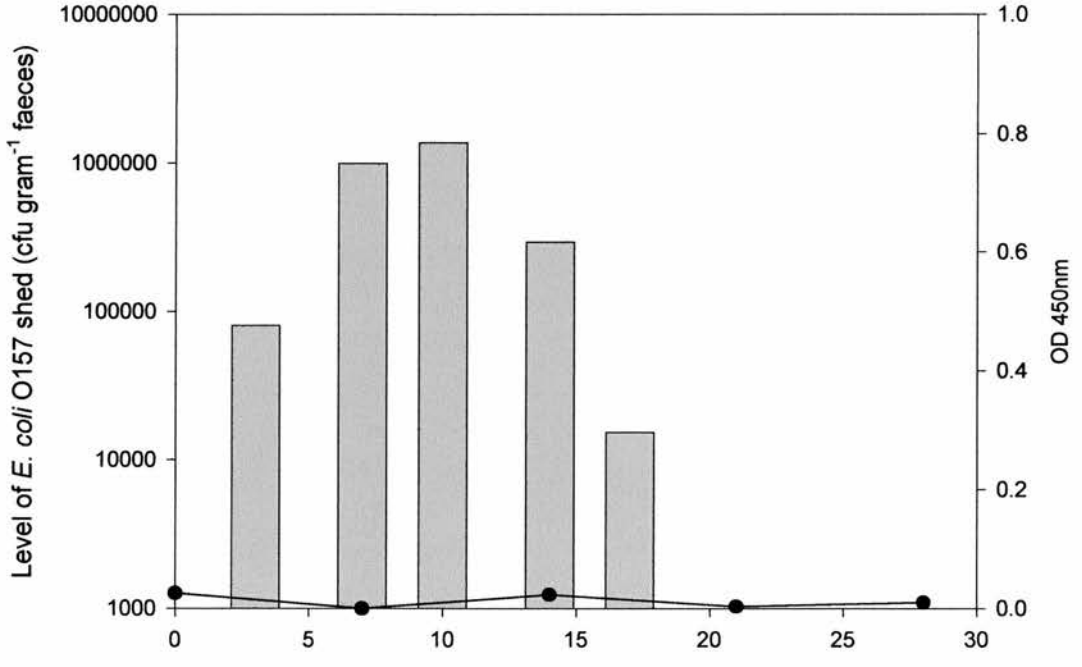


Figure A4.32: Calf c313. Legend as A4.29

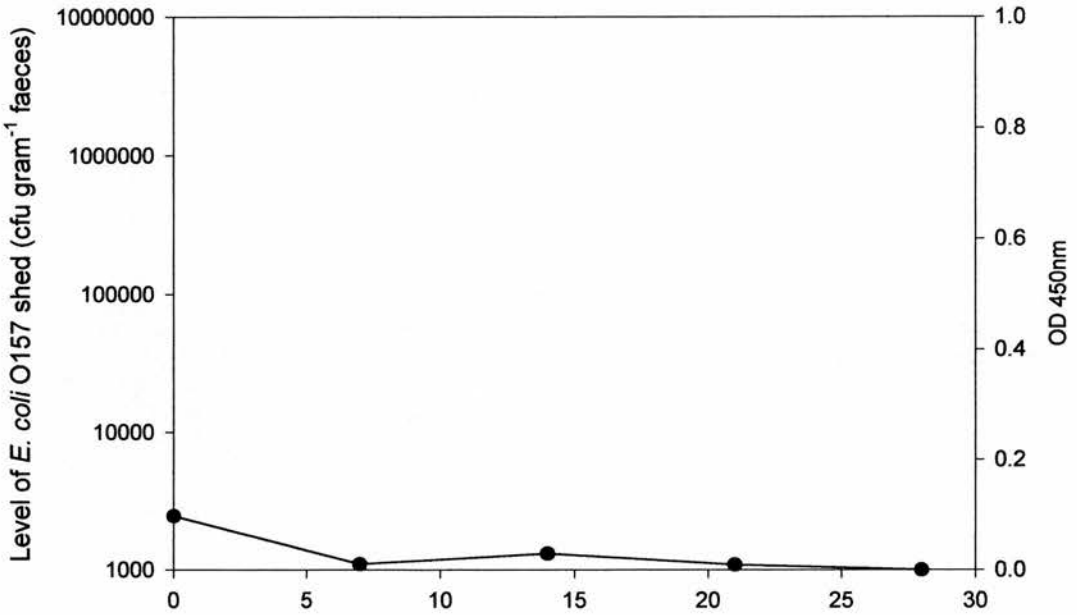


Figure A4.33: Calf c322. Legend as A4.29

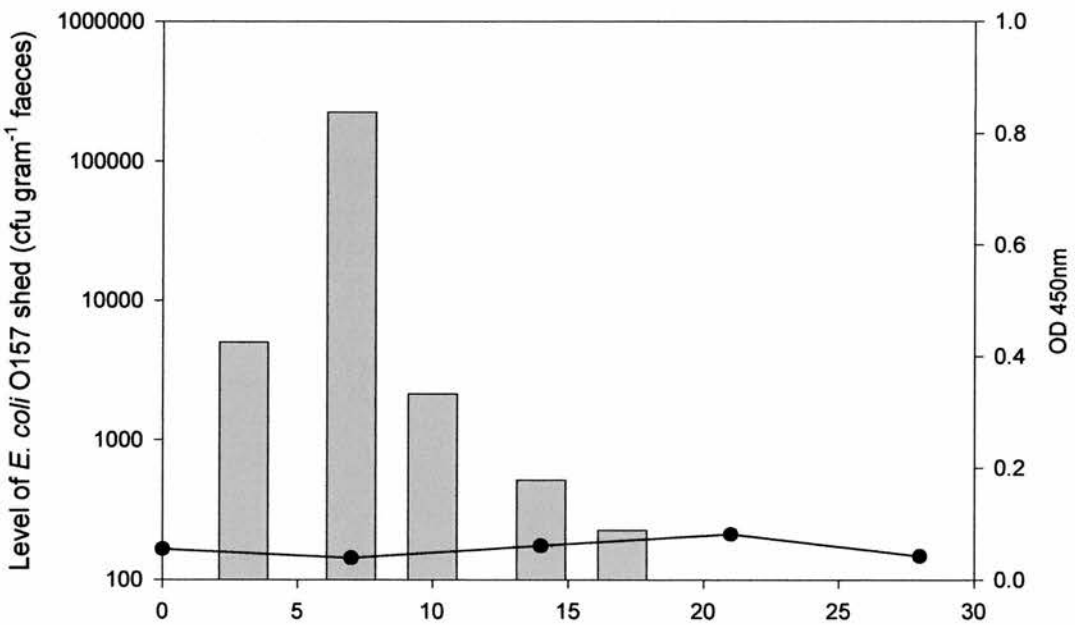


Figure A4.34: Calf c324. Legend as A4.29

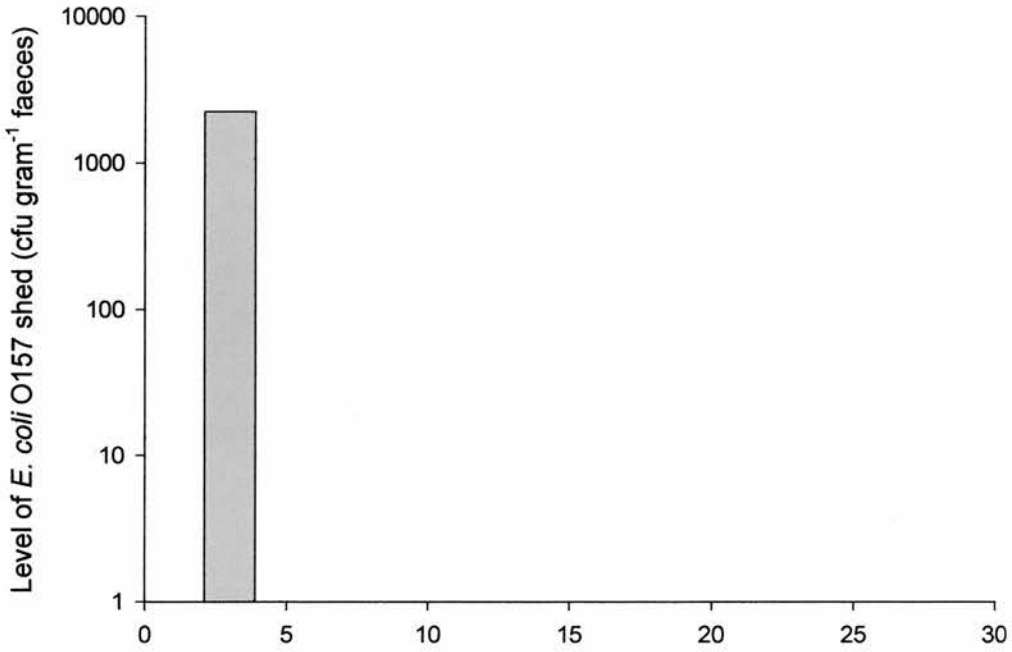
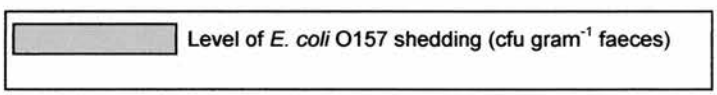


Figure A4.35. Calf c296. Level of *E. coli* O157 shed from an experimentally infected calf over a four-week period



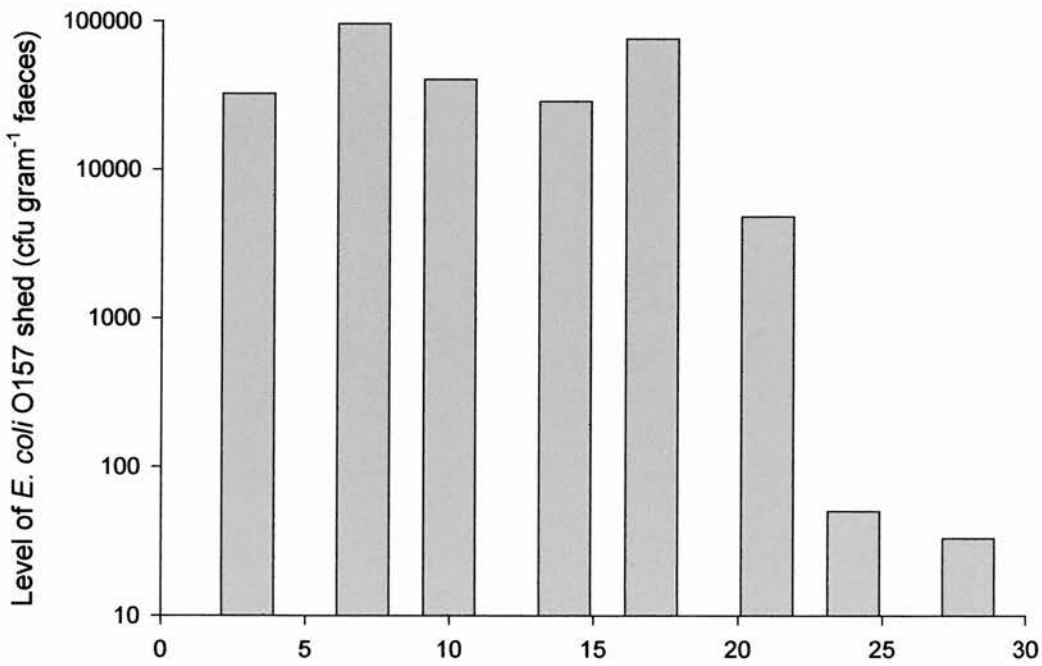


Figure A4.36. Calf c303. Legend as A4.35

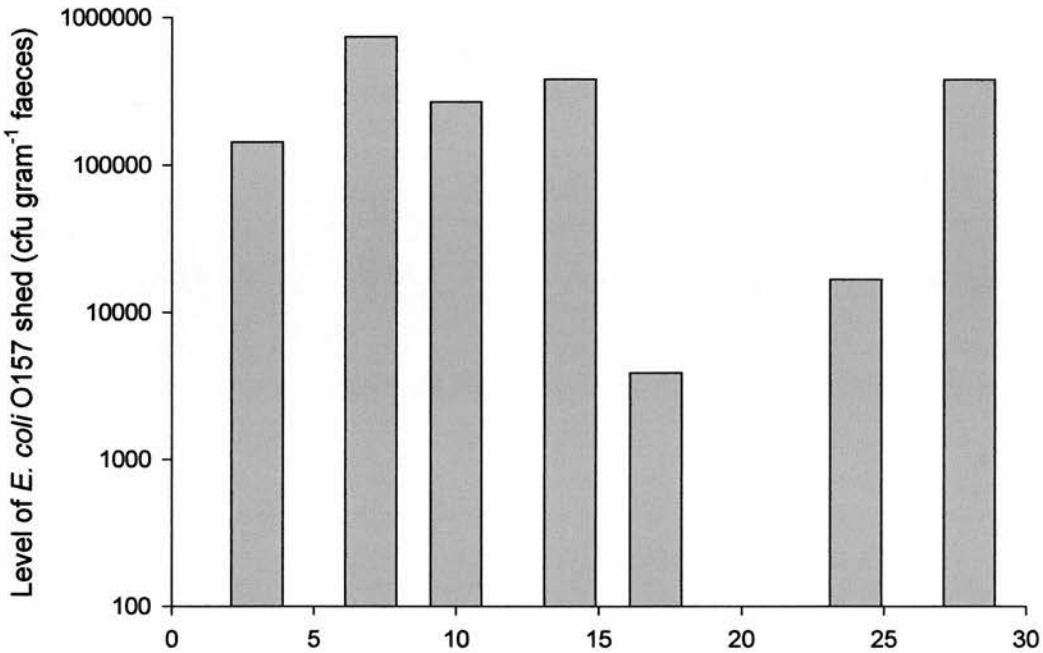


Figure A4.37. Calf c310. Legend as A4.35